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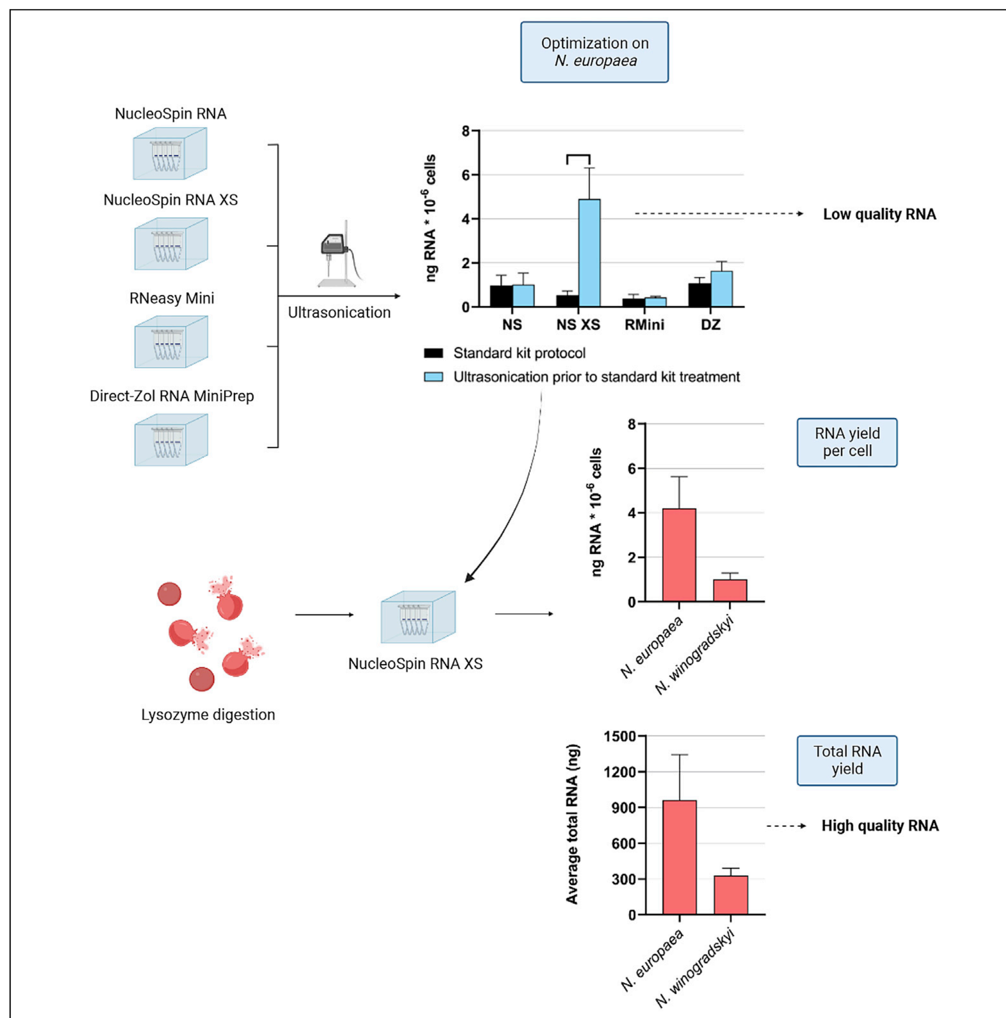

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Highlights

Lysis through ultrasonication results in a high RNA yield but in a low RNA quality

Enzymatic lysis using lysozyme provides high-yield, high-quality RNA samples

RNA from low-biomass bacterial samples is suitable for downstream RNA-seq

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Optimization of RNA extraction for bacterial whole transcriptome studies of low-biomass samples

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SUMMARY

We developed a procedure for extracting maximal amounts of high-quality RNA from low-biomass producing (autotrophic) bacteria for experiments where sample volume is limited. Large amounts of high-quality RNA for downstream analyses cannot be obtained using larger quantities of culture volume. The performance of standard commercial silica-column based kit protocols and these procedures amended by ultrasonication or enzymatic lysis were assessed. The ammonium-oxidizing *Nitrosomonas europaea* and nitrite-oxidizing *Nitrobacter winogradskyi* were used as model organisms for optimization of the RNA isolation protocol. Enzymatic lysis through lysozyme digestion generated high-quality, high-yield RNA samples. Subsequent RNA-seq analysis resulted in qualitative data for both strains. The RNA extraction procedure is suitable for experiments with volume and/or biomass limitations, e.g., as encountered during space flight experiments. Furthermore, it will also result in higher RNA yields for whole transcriptome experiments where sample volume and/or biomass was increased to compensate the low-biomass characteristic of autotrophs.

INTRODUCTION

Whole transcriptome sequencing provides valuable information on the physiological response of microorganisms to adjustments in the external environment. Differentially expressed genes and their associated pathways are identified in different conditions, leading to understanding of the response of the bacteria to environmental changes. An important requirement for successful RNA sequencing (RNA-seq) is the availability of pure RNA in sufficient quantities and with minimal transcript degradation. Low quantity RNA samples result in low coverage sequencing results and the absence of transcripts that are lowly expressed. In this case, deeper sequencing is required to avoid suboptimal results (Adiconis et al., 2013), which comes at a higher cost price. Available RNA should be maximized to provide enough reads, ensuring that the transcriptome of the bacteria is sequenced as completely as possible. Another important factor to take into consideration is RNA degradation. Low sample quality can result in 3′ end bias where short reads are generated from 3′ ends of a transcript but significantly decrease toward the 5′ end of transcripts, resulting in 3′ end enrichment (Wan et al., 2012). Finally, degradation can have an impact on measured gene expression levels and the ability to detect low-expression genes (Romero et al., 2014). High-quality RNA is therefore important to ensure accurate and optimal mapping of RNA reads to the reference genome.

Obtaining sufficient RNA is usually not an issue when targeting heterotrophic bacterial strains or other organisms characterized by a high biomass production. However, in the case of specific autotrophic bacteria which are characterized by low biomass concentration in axenic suspension cultures (OD_{600} = 0.025–0.070), RNA yield is limited. This issue is easily resolved in current protocols by extracting RNA from large volume samples and from chemostat cultures, where higher biomass concentrations are reached (Perez et al., 2015; Sayavedra-Soto et al., 2015; Sedlacek et al., 2020), in combination with additional mechanical lysis steps such as bead-beating (Miyamoto et al., 2018; Sayavedra-Soto et al., 2015) or ultrasonication (Sedlacek et al., 2020). However, in some cases, the use of large volume cultures is not possible. For example, for space experiments, payload is limited because of high costs and restrictions imposed by space agencies, leading to small sample volumes available for data generation. Also, in studies where multiple samples have to be taken for different experiments, sample volume could also be a limiting factor because of the need for adequate amounts of culture volume for all experiments. In this case, larger amounts of volume could be needed, for example, proteomic and/or metabolomics analyses, leaving less volume for a whole

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Table1. Overview of RNA extraction kits from commercial vendors

Extraction kit	Abbreviation	Vendor
NucleoSpin® RNA	NS	Machery-Nagel Inc., Allentown, PA, USA
NucleoSpin® RNA XS	NS XS	Machery-Nagel Inc., Allentown, PA, USA
RNeasy Plus Mini	RMini	QIAGEN Inc., Hilden, Germany
RNeasy Plus Micro	RMicro	QIAGEN Inc., Hilden, Germany
Direct-zol™ RNA MiniPrep	DZ	Zymo research Corp., Irvine, CA, USA

transcriptome study of the same culture. In such research with similar volume limitations, RNA yield has to be optimized by maximization of cell lysis whereas degradation of RNA molecules is kept at a minimum. Moreover, all RNA from lysed cells needs to be isolated from the sample to ensure no information is lost for further analyses.

During development of this methodology, several commercial silica-column based RNA extraction kits were tested whereas phenol-chloroform RNA extraction methods were not included. Although these methods generally provide a higher RNA yield than silica-column based methods, they are more prone to contain contaminating substances that can have a detrimental effect on the downstream RNA-seq analysis (Nwokeoji et al., 2016; Toni et al., 2018). Moreover, these methods are usually time-consuming and use hazardous reagents (Nwokeoji et al., 2016). The quality of an RNA sample can be quantified with an RNA Integrity Number (RIN), a quantification method that rates RNA quality on a scale from 1 to 10 (1 = completely degraded RNA, 10 = most intact RNA) (Jahn et al., 2008; Schroeder et al., 2006). High quality RNA, i.e., a RIN of 8 or higher, is a prerequisite for successful RNA sequencing (Hitzemann et al., 2013; Jahn et al., 2008; Romero et al., 2014; Sigurgeirsson et al., 2014). Another important factor to take into account is that the RIN scores of samples from different conditions in one experiment should be comparable to avoid biases based on differential quality levels (Reiman et al., 2017). However, it is important to mention that some studies also confirmed the presence of good quality mRNA sequences in samples with low RIN scores and make a case that these samples could also be included in transcriptome research (Puchta et al., 2020; Reiman et al., 2017; Romero et al., 2014). Next to RIN-values, Transcript Integrity Number (TIN)-values are another measurement for the extent of RNA degradation. These values can only be determined from RNA-seq data. It determines the degree of degradation of the transcripts and thus at the mRNA level, whereas the RIN-value relies heavily on 16 and 23S rRNA quality. Hence, it is a useful parameter to assess transcript quality after RNA-seq (Wang et al., 2016).

In this publication, a procedure was developed for low-biomass-producing nitrifying bacteria where sample volume is limited. For optimization, the autotrophic nitrifying bacteria *Nitrosomonas europaea* and *Nitrobacter winogradskyi* were selected as test organisms. These Gram-negative bacteria oxidize ammonium to nitrite or nitrite to nitrate, respectively, to provide energy for CO_2 carbon fixation. Because of this metabolism, both are characterized by slow growth and a low final biomass in stationary phase, in axenic suspension cultures. An optimized procedure for low volume *N. europaea* cultures was first developed and subsequently validated on axenic cultures of *N. winogradskyi*. Variations in the optimized protocol for *N. europaea* were required for the development of a procedure that also adequately lysed *N. winogradskyi* cells while retaining efficacy on *N. europaea* cells. Validation of the procedure was also evaluated on heterotrophic, high biomass producing *Comamonas testosteroni* cultures.

Table 2. Overview of buffer composition and volume added for each RNA extraction kit

Extraction kit	Lysis buffer name	Chaotropic agent	Reducing agent	Volume added (μL)
NS	RA1	Guanidine thiocyanate	TCEP	350
NS XS	RA1	Guanidine thiocyanate	TCEP	100
RMini	RLT	Guanidine thiocyanate	BME	600
RMicro	RLT	Guanidine thiocyanate	BME	350
DZ	TRIzol®	Guanidine thiocyanate	–	300

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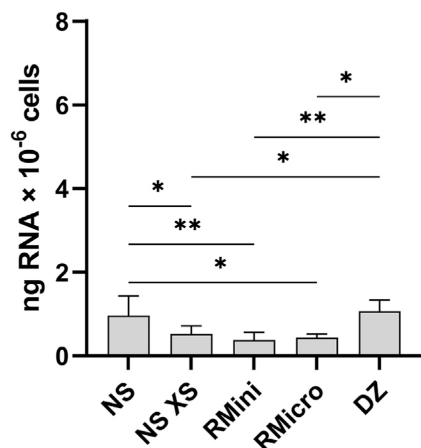

Figure 1. RNA yield from *N. europaea* cultures with different commercial RNA extraction kits

Amount of RNA in ng per 10^6 cells from 5 mL *N. europaea* cultures extracted with commercial silica-column based RNA extraction kits (NS = NucleoSpin RNA, NS XS = NucleoSpin RNA XS, RMicro = RNeasy Micro, DZ = Direct-Zol RNA MiniPrep).

Data is presented as mean $\pm$ SD ($n \geq 4$). Analysis of Variance (ANOVA) and post-hoc Tukey's tests were performed to identify significant differences between procedures: $^*p < 0.05$, $^{**}p < 0.01$ (See also Table S1).

RESULTS AND DISCUSSION

Comparison of commercial silica-column based RNA extraction kits

In a first step toward the optimization of RNA extraction on low-biomass producing cultures, the standard protocols of several commercial RNA extractions kits listed in Table 1 were applied to *N. europaea* samples. The exact composition of the lysis buffers provided with these kits is proprietary and undisclosed by the manufacturer. An overview for each commercial kit is provided in Table 2. For all RNA extraction kits, the lysis buffer contains guanidine thiocyanate as a chaotropic agent. Also, the volume of the lysis buffer differs between the kits. In the standard protocol of the NS XS kit, 100 μL of lysis buffer 'RA1' is added, whereas the addition of 350 μL RA1 is required in the NS standard procedure. Similarly, for the RMicro kit, 350 μL of 'RLT' lysis buffer is added compared to 600 μL RLT with the RMini kit. Finally, 300 μL of triZOL was used before manipulation of the sample with the DZ kit. An amount of reducing agent TCEP (tris(2-carboxyethyl)phosphine) or β-mercaptoethanol (BME) is added to the lysis buffer for the NS kits or the RNeasy kits, respectively. These reducing agents contribute to the inactivation of RNases present in the solution of the buffer and the resuspended cell pellet. Total RNA yields were quantified for each kit (Figure 1).

NucleoSpin RNA and Direct-Zol RNA MiniPrep isolated significantly more RNA (276.35 $\pm$ 131.27 ng; 0.96 $\pm$ 0.47 ng $\times$ 10^{-6} cells, and 313.9 $\pm$ 76.55 ng; 1.07 $\pm$ 0.26 ng $\times$ 10^{-6} cells, respectively) from the samples ($p < 0.05$) as opposed to the other RNA extraction kits. NucleoSpin RNA XS (142.10 $\pm$ 51.72 ng; 0.52 $\pm$ 0.20 ng $\times$ 10^{-6} cells), RNeasy Plus Mini (105.6 $\pm$ 50.72 ng; 0.37 $\pm$ 0.19 ng $\times$ 10^{-6} cells) and RNeasy Plus Micro (124.8 $\pm$ 24.07 ng; 0.44 $\pm$ 0.09 ng $\times$ 10^{-6} cells) all extracted equivalent amounts of RNA. When looking at total RNA yield (ng), downstream RNA-seq might be achievable with these amounts of RNA but they remain on the lower side of required total RNA yield for most companies that perform RNA-seq. Moreover because the concentration of all elutions was lower than 10 ng/μL,a RIN-value could not be determined. The latter is a critical guideline to ensure proper high-quality RNA-seq. An overview of all tested procedures can be found in Table S1.

Because all standard commercial kit procedures generated low amounts of RNA, mechanical or enzymatic lysis steps were amended to the standard kit protocol in an attempt to enhance RNA yield.

The effect of ultrasonication on RNA yield and quality from *N. europaea* cultures

To increase cell lysis, samples were resuspended in the lysis buffer of the respective RNA isolation kit and subjected to ultrasonication pulses (3 × 20 s, 40% amplitude, 1 cycle, in between 1 min on ice) (Figure 2) before proceeding with the standard kit protocol. Because the RNeasy Plus Mini and Micro kits previously yielded very similar results in the standard procedures, the RNeasy Plus Micro kit was omitted in this test.

An almost 10-fold increase of total extracted RNA (1539.86 $\pm$ 596.81 ng; 4.90 $\pm$ 1.41 ng $\times$ 10^{-6} cells) was observed using the NucleoSpin RNA XS when the standard protocol was preceded by ultrasonication. Meanwhile, no significant effects on RNA yield were observed in the NucleoSpin RNA, RNeasy Mini Plus and Direct-Zol kits when combined with ultrasonication (283.10 $\pm$ 143.13 ng; 1.01 $\pm$ 0.52 ng $\times$ 10^{-6} cells,

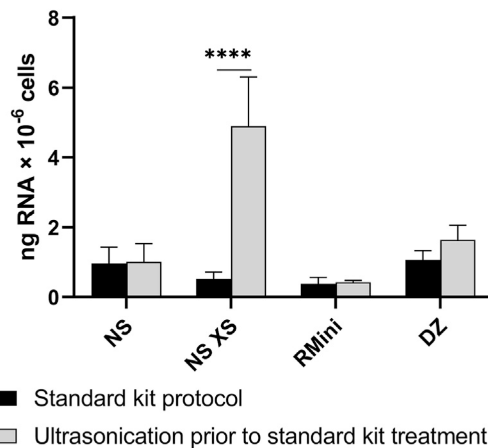

Figure 2. The effect of ultrasonication on RNA yield and quality from *N. europaea* cultures using different commercial silica-column based kits

Amount of RNA in ng per 10^6 cells from 5 mL *N. europaea* cultures extracted with commercial silica-column based RNA extraction kits (NS = NucleoSpin RNA, NS XS = NucleoSpin RNA XS, RMicro = RNeasy Micro, DZ = Direct-Zol RNA MiniPrep) with and without prior ultrasonication step (see STAR Methods). Data is presented as mean $\pm$ SD ($n \geq 3$). A t-test was performed between different treatments for each kit: ****$p < 0.0001$. (See also Table S1).

0.43 $\pm$ 0.05, and 123.2 $\pm$ 14.40 ng; 1.64 $\pm$ 0.42 ng × 10^{-6} cells, respectively) in comparison to the standard kit treatment. This data clearly showed a strong improvement of RNA yield when cells are lysed by ultrasonication before sample manipulation with the NucleoSpin RNA XS kit. RIN-values were also determined for these samples (where the RNA concentration was higher than 10 ng/μL). Unfortunately, low numbers were observed (RIN-value between 2 and 5), indicating strong RNA degradation.

In an attempt to minimize potential RNA degradation following ultrasonication, several approaches were investigated by adapting either the composition or volume of the lysis buffer of the NucleoSpin RNA XS kit in which the cell pellet is resuspended, or by adapting the ultrasonication procedure. First, different concentrations of reducing agent TCEP in the lysis buffer (RA1) were applied; both reagents are provided with the kit. In the standard protocol, 2 μL of 140 mg/mL TCEP is added to 100 μL RA1 before resuspending the pellet in this solution and continuing with ultrasonication. In this setup, 2, 3 or 4 μL of TCEP was added to 100 μL of RA1 and the effect on RNA quality was evaluated (Figure 3A). Second, the pellet was resuspended in different volumes of RA1 whereas keeping TCEP:RA1 constant at 2 μL TCEP for every 100 μL of RA1 (Figure 3B). Finally, we investigated whether changes to the ultrasonication step itself affected RNA quality by either decreasing the amplitude or the duration of the ultrasonication burst (Figure 3C).

Adapting the TCEP to RA1 ratio in lysis buffer did not lead to an effect on RNA yield nor were any increases in RNA quality observed. When RA1 volume was increased, RNA yield dropped significantly. As previously mentioned, RNA yield from cells resuspended in 100 μL RA1 and ultrasonicated was 1539.86 $\pm$ 596.81 ng or 4.90 $\pm$ 1.41 ng × 10^{-6} cells. For 200 μL RA1 and 300 μL RA1, the values decreased to respectively 151.68 $\pm$ 50.33 ng or 0.41 $\pm$ 0.14 ng × 10^{-6} cells, and 89.56 $\pm$ 37.94 ng or 0.24 $\pm$ 0.10 ng × 10^{-6} cells. Finally, when decreasing the ultrasonication pulse duration and/or intensity, RNA yield also decreased, indicating incomplete lysis of the bacteria. These results demonstrate that ultrasonication adequately lyses all cells in suspension provided that the intensity is high enough (3 × 15 or 20 s, 40% amplitude, 1 cycle, cooled for 1 min on ice in between ultrasonication bursts) and the sample volume low enough (100 μL RA1 buffer) because no lysis was observed at higher volumes in which cells were resuspended. However, this type of mechanical lysis also extensively damages RNA molecules in all cases where a high yield was obtained. These low RIN-values were not reported by a study in which ultrasonication (2 × 30 s, 35% amplitude, 1 cycle, 30 son ice) was used to lyse *N. europaea* cells for RNA extraction (Sedlacek et al., 2020). Because higher culture volumes were used, adequate amounts of RNA could be extracted with a lower ultrasonication intensity, hence preserving RNA quality. In our case, a similar duration and intensity of ultrasonication (3 × 20 s, 35% amplitude, 1 cycle, 1 min on ice) resulted in very low RNA yields but a high RIN-value (7.7, only 1 value could be determined because only this sample had an RNA concentration greater than 10 ng/μL) was observed, thus confirming that this ultrasonication intensity does not contribute to RNA degradation, but does not lyse all cells in suspension.

The effect of enzymatic lysis on RNA yield and quality from *N. europaea* cultures

Since mechanical lysis does not meet the requirements for optimal downstream processing of RNA because of substantial sample degradation, the efficiency of enzymatic lysis instead of mechanical lysis

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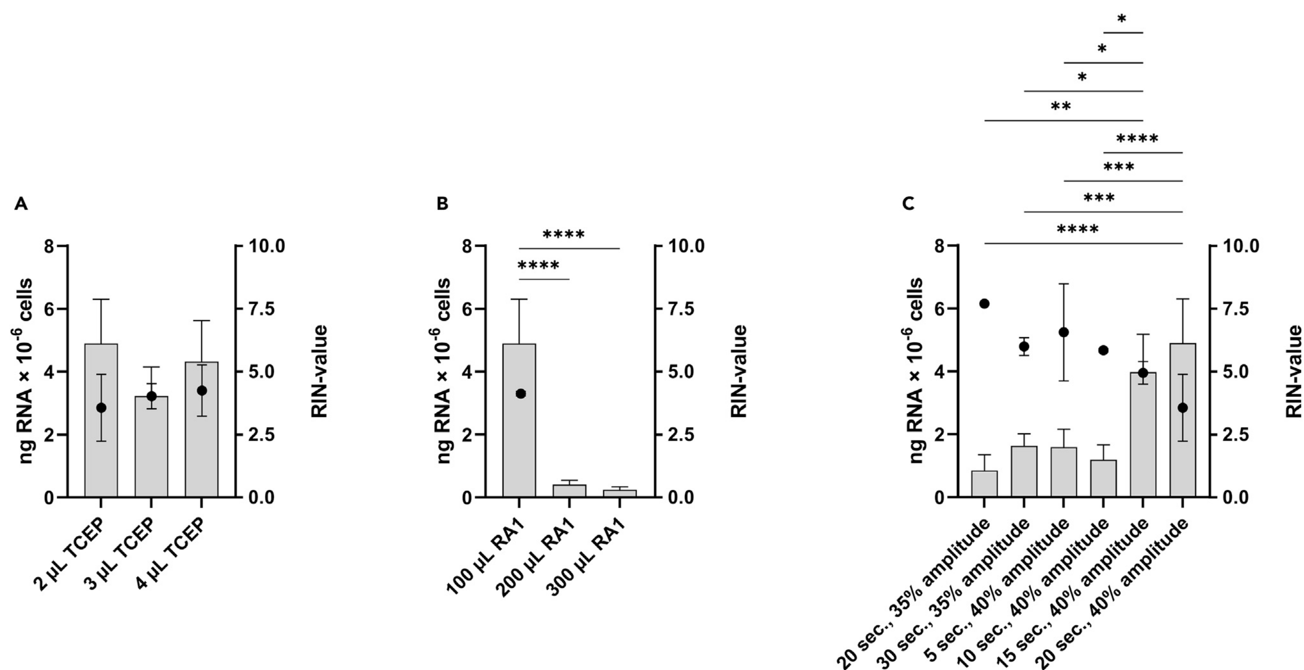

Figure 3. The effect of ultrasonication on RNA yield and quality from *N. europaea* cultures

Amount of RNA in ng per 10^6 cells from 5 mL *N. europaea* cultures extracted with NucleoSpin XS RNA kit with modifications to the standard protocol and amended by an ultrasonication treatment (A and B), and from the standard kit protocol preceded by different ultrasonication treatments (C). RIN-values of each procedure (if available) are indicated by black dots. Data is presented as mean ± SD (n ≥ 4). ANOVA and post-hoc Tukey's tests were performed to identify significant differences of RNA yields within each procedure: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (See also Table S1).

before treatment with the NucleoSpin RNA XS kit was investigated. For Gram negative bacteria such as *N. europaea* and *N. winogradskyi*, 1 mg/mL lysozyme solution is usually effective in combination with a 1x TE buffer at a pH of 8.00. In this buffer solution, EDTA disrupts the outer membrane (Haque and Russell, 1974), which allows the lysozyme to approach the cell wall and digest the peptidoglycan. However, to investigate whether lysis is complete, higher lysozyme concentrations (2 mg/mL, 3 mg/mL) were also included in the experiment (Figure 4A). For all 3 concentrations, the RNA yield was strongly increased compared to the standard kit protocol (1 mg/mL: 947.79 ± 351.43 ng or 4.21 ± 1.41 ng × 10^{-6} cells; 2 mg/mL: 749.49 ± 25.78 ng or 3.40 ± 0.07 ng × 10^{-6} cells; 3 mg/mL: 705.24 ± 377.41 or 3.21 ± 1.74 ng × 10^{-6-} cells) but no significant differences were observed between the different concentrations. As expected, 1 mg/mL lysozyme caused sufficient cell lysis. Moreover, the observed RIN-values were consistently greater than 8. BioAnalyzer electropherograms consistently show sharp 23 and 16S fragment peaks on which RIN-value calculation is based (Figure 5B). On the other hand, RNA extracted using ultrasonication as pretreatment did not show sharp peaks (Figure 5A), indicating fragmentation of these rRNA molecules. Thus, by using lysozyme pretreatment, all requirements for a qualitative downstream RNA-seq analysis were met.

Additional potential optimization steps were tested subsequently. Extraction with lysozyme treatment followed by increased volumes of RA1 buffer additions from 100 µL to 200 and 300 µL provided significantly higher RNA yields for both 200 and 300 µL RA1 compared to the standard 100 µL RA1 as described in the NucleoSpin RNA XS kit protocol (Figure 4). Hence, addition of 200 µL RA1 following lysozyme digestion was used in downstream procedures. Finally, both a shorter (5 min) and a longer (30 min) incubation time of a 1 mg/mL lysozyme solution followed by addition of 200 µL RA1 yielded similar RNA amounts (Figure 4). The protocol with 15 min of incubation time at 37°C and 200 µL RA1 buffer was also validated on *N. europaea* cultures grown in SUSS medium, where OD_{600} is typically lower after 7 days compared to *N. europaea* grown in ATCC 2265 medium. The SUSS medium is a urine salts matrix in which both *N. europaea* and *N. winogradskyi* can grow, if their respective nitrogen source (NH_4^+ or NO_2^-) is added to the medium composition. Nonetheless, the NucleoSpin RNA XS kit procedure with the lysozyme treatment

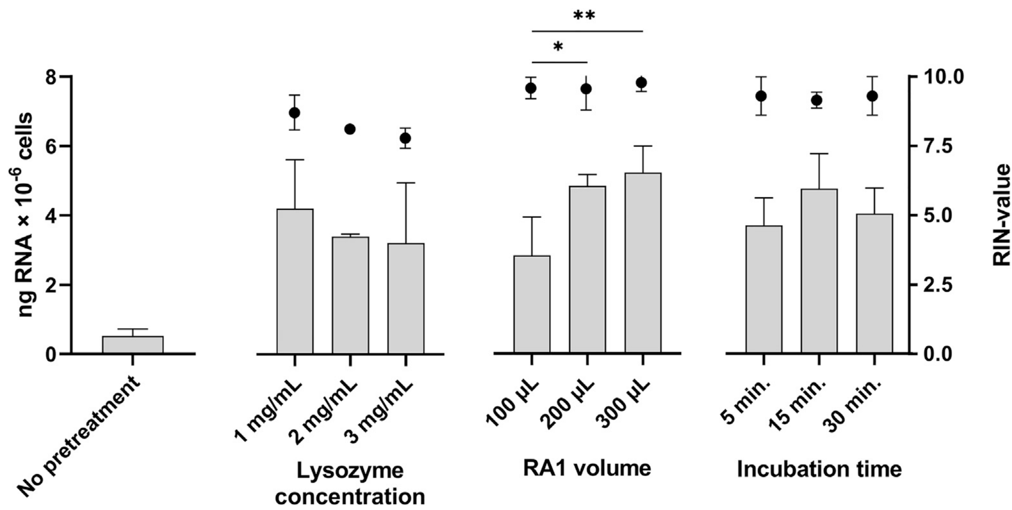

Figure 4. Effect of enzymatic lysis on RNA yield and quality from *N. europaea* cultures

Amount of RNA in ng per 10^6 cells from 5 mL *N. europaea* cultures extracted with NucleoSpin RNA XS kit preceded by enzymatic digestion with lysozyme solution. The effect on RNA yield and quality of different lysozyme concentrations, different volumes of RA1 buffer after lysozyme digestion and different lysozyme incubation times are shown. RIN-values of each procedure (if available) are indicated by black dots. Data is presented as mean ± SD (n ≥ 4). ANOVA and post-hoc Tukey's tests were performed to identify significant differences within each procedure and without lysozyme pretreatment ($p < 0.05$): *$p < 0.05$, **$p < 0.01$. All samples tested significantly different ($p < 0.01$) compared to RNA extractions without lysozyme pretreatment (See also Table S1).

resulted in qualitative RNA samples of adequate yield from *N. europaea* grown in SUSS medium (665.89 ± 241.66 ng; 3.54 ± 0.50 ng × 10^{-6} cells; RIN = 9.9 ± 0.1) (Table S1).

The effect of enzymatic lysis on RNA yield and quality from *N. winogradskyi* cultures

The performance of the NucleoSpin XS RNA kit with preceded by 1 mg/mL lysozyme digestion and the addition of 200 µL RA1 buffer was further examined on cultures of *N. winogradskyi*. This strain proved to be more difficult to lyse by the lysozyme treatment. However, the extraction protocol yielded adequate amounts of RNA (330.00 ± 60.00 ng; RIN = 8.1 ± 0.2) (Figure 6A), albeit less RNA yield per cell (1.01 ± 0.27 ng × 10^{-6} cells) than from *N. europaea* cultures, as expected. Although adequate amounts of RNA were obtained with this protocol, we investigated whether we could further optimize the RNA yield per cell from *N. winogradskyi* cultures. We tested higher lysozyme concentrations Figure 6B) and longer

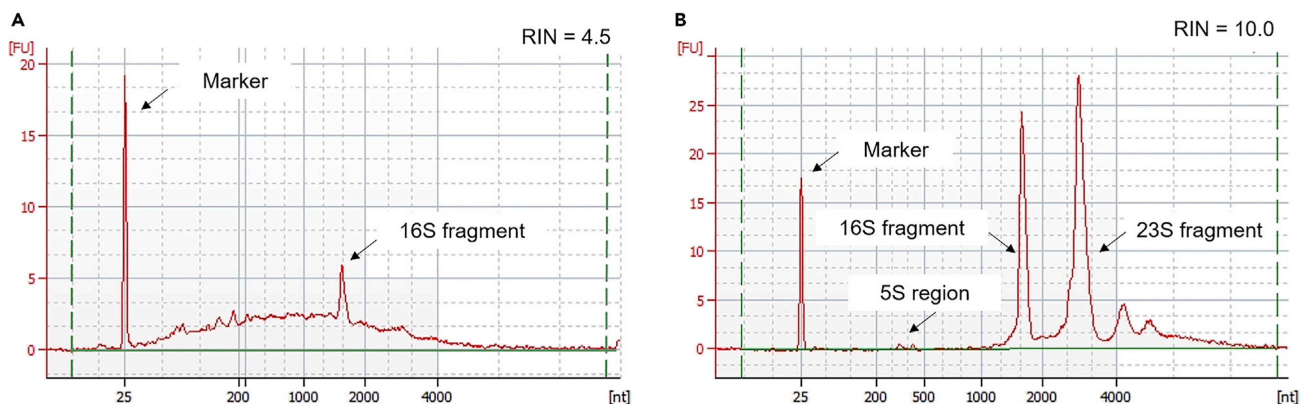

Figure 5. Examples of electropherograms generated from RNA samples with the Agilent BioAnalyzer 2100

(A) represents an RNA sample extracted with the NS XS kit after ultrasonication pretreatment (3 × 20 s, 40% amplitude, 1 cycle, in between 1 min on ice) with a RIN of 4.5.

(B) represents an RNA sample extracted with the NS XS kit after pretreatment with a 1 mg/mL lysozyme solution and 15 min of incubation at 37°C with a RIN of 10.0.

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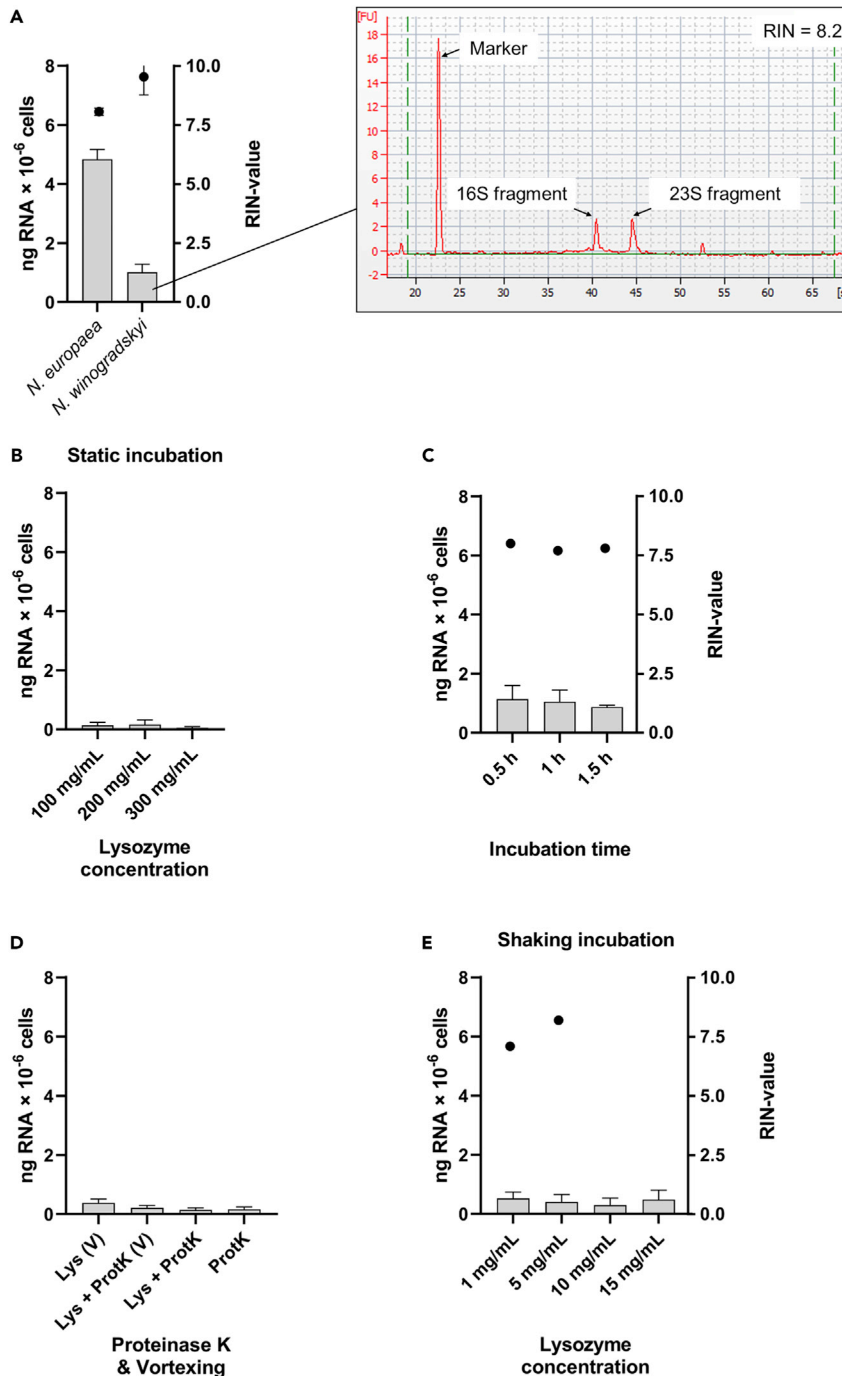

Figure 6. Effect of enzymatic lysis on RNA yield and quality from *N. winogradskyi* cultures

Amount of RNA in ng per 10^6 cells from 5 mL *N. winogradskyi* cultures extracted with the NucleoSpin RNA XS kit preceded by enzymatic digestion with a 1 mg/mL lysozyme solution, including an example of a BioAnalyzer electropherogram of one of the samples and compared to RNA yield extracted from *N. europaea* cultures (A). The protocol on *N. winogradskyi* cultures using higher lysozyme concentrations (B), with different incubation times with 1 mg/mL lysozyme solution (C), with the addition of Proteinase K (ProtK) to a 15 mg/mL lysozyme solution and/or vortexing (indicated by V) (D) and with varying lysozyme concentrations during shaking incubation at 1,400 rpm for 15 min (E). RIN-values of each procedure (if available) are indicated by black dots. In all cases except for (A), RIN-values represent the average of less than 3 available

Figure 6. *Continued*
values because not all samples had an RNA concentration greater than 10 ng/μL (with the exception of graph A). Data is presented as mean ± SD ($n \geq 3$). ANOVA and post-hoc Tukey's tests were performed to identify significant differences between procedures ($p < 0.05$). (See also Table S1).

incubation times (Figure 6C). Also, the effect of a combined enzymatic step with the addition of 10μL of a 20 mg/mL proteinase K solution was assessed (according to (Giannoukos et al., 2012)). Moreover, this step was also done in combination with agitating the solutions during treatment (Figure 6D). Finally, the results of continuously shaking at 1,400 rpm during incubation in 1, 5, 10, or 15 mg/mL lysozyme solution at 37°C were also assessed (Figure 6E).

None of these variations markedly improved RNA yield. On the contrary, most variations resulted in a decrease in RNA yield. In one case, high lysozyme concentration treatment barely extracted any RNA, with RNA concentrations ranging from 1–3 ng/μL (30–90 ng of total RNA or 0.05–0.14 ng × 10^{-6-} cells). No significant differences were observed compared to the protocol optimized for *N. europaea* except for the use of 300 mg/mL, which yielded significantly less RNA in comparison. Yields generated during 0.5 h or 1 h incubation periods (390.00 ± 196.72 ng; 1.14 ± 0.46 ng × 10^{-6} cells, and 300.00 ± 60.00 ng; 0.87 ± 0.06 ng × 10^{-6} cells, respectively) were significantly higher than yields from 100, 200, or 300 mg/mL lysozyme treatments (50.00 ± 34.64 ng; 0.14 ± 0.09 ng × 10^{-6} cells, 50.00 ± 45.83 ng; 0.16 ± 0.16 ng × 10^{-6}cells, and 20.00 ± 17.32 ng; 0.05 ± 0.04 ng × 10^{-6} cells). These observations infer that high lysozyme concentrations could have an inhibitory effect on its activity.

Based on the aforementioned results, a final protocol was established where cell pellets are resuspended in 200 μL of a 1 mg/mL lysozyme solution, incubated for 15min at 37°C, followed by treatment with the NucleoSpin RNA XS kit where 200 μL RA1 buffer with 4 μL TCEP is added to the lysozyme mixture after digestion. The validity of the technique was also confirmed on the heterotrophic, high-biomass producing *C. testosteroni*. The protocol generated an RNA yield of 6550.00 ± 905.15 ng of RNA with an average RIN-value of 8.6 ± 2.0 (Table S1).

Validation of RNA quality for whole transcriptome analysis

Usually, assessing RNA quality through RIN calculation is sufficient to determine whether a sample's quality is adequate for downstream RNA-seq. Nevertheless, to further confirm our results, additional tests were performed on the quality of the sequencing results of 200 ng of extracted RNA, and on the mapping of raw reads to the reference genome of either *N. europaea* or *N. winogradskyi*. The quality analysis after genome alignment provides a deeper level of sample quality validation than RIN value alone An overview of the quality parameters is given in Table 3.

Raw reads passed a QC check using FastQC, which is a common practice to determine if the RNA-seq procedure was successful. The average per base sequence quality scores for *N. europaea* and *N. winogradskyi* samples were 35.85 and 35.63, respectively. Moreover, they remained equivalent along the position in the read.

In the scope of this paper, additional quality checks were performed after mapping of the paired-end reads. In the case of *N. europaea* samples, an average mapping quality score of 34.61 was reached while this score was 33.85 for RNA samples originating from *N. winogradskyi* cultures. In both cases, this implies that the average probability that a base is mapped correctly is greater than 99.9% (Li et al., 2008).

For both strains, coverage across genes was also analyzed (Figure 7A) and the TIN-values of each transcript were determined (Figure 7B). The coverage across genes profile for *N. europaea* showed an even coverage. These profiles showed a 5′ end bias for RNA extracted from *N. winogradskyi*. The profiles of all three biological replicates of this strain are very similar, hence displaying good reproducibility. It is possible that this is a general coverage of genes profile for *N. winogradskyi* RNA. This could be a

Table 3. Overview of sequencing and mapping quality parameters

Strain	Average # of reads	Average per base sequence quality	Average mapping quality
N. europaea	43.7×10^6	35.85	34.61
N. winogradskyi	39.2×10^6	35.68	33.85

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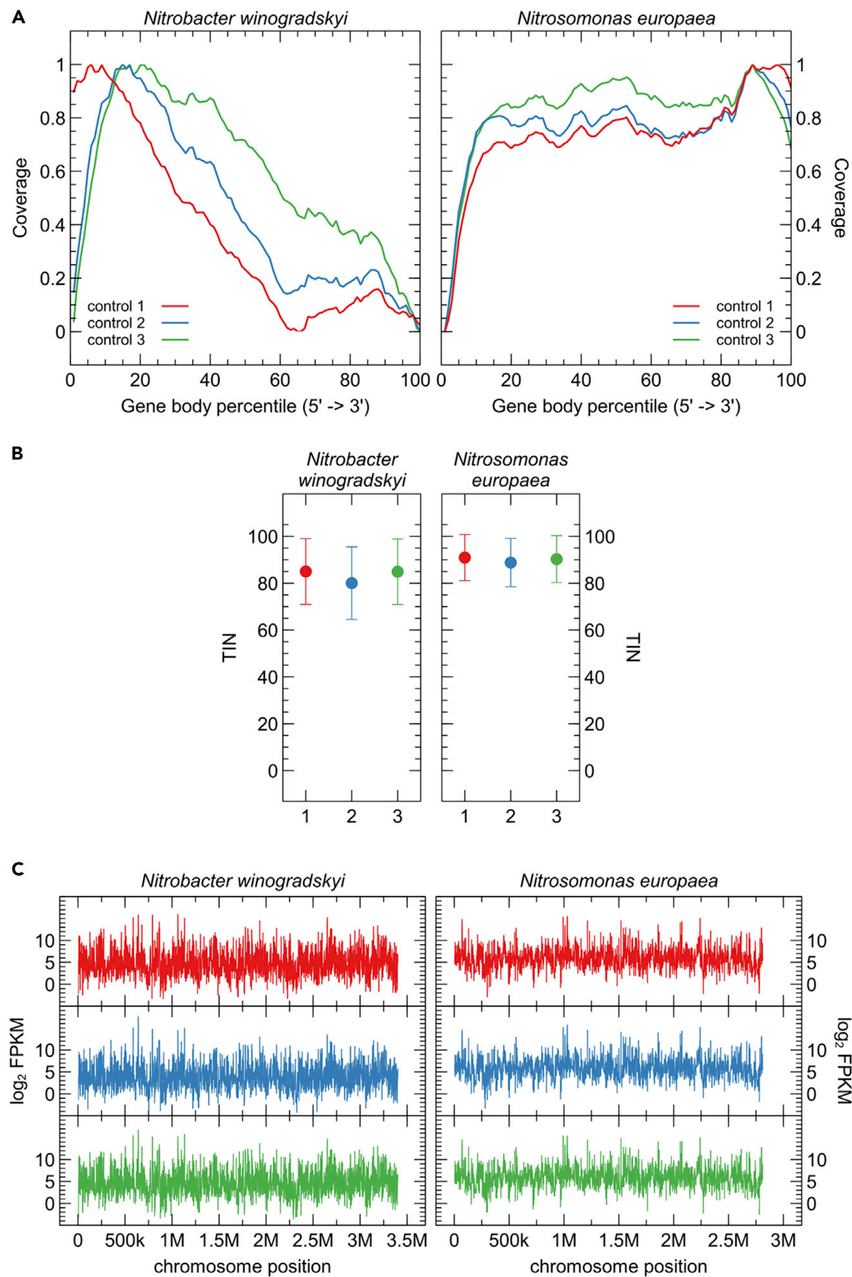

Figure 7. Quality assessment of mapped reads generated by sequencing RNA samples from the optimized protocol

(A–C) Coverage over genes, (B) Average TIN-value and (C) $\log_2$ FPKM mapped across the chromosome per biological replicate.

consequence of RNA turnover or the rate of production of mRNA molecules inside the cell. However, because of the reproducibility of the profile, it is unlikely that downstream differential gene expression analysis will be affected. In addition, because cDNA libraries from RNA of both strains were constructed using the same kit, it is hard to attribute the 5′-end bias to the library preparation step. Moreover, average TIN-values for both strains were high, although *N. europaea* values were higher compared to *N. winogradskyi* TIN-values. For both strains, these values indicate low transcript degradation and thus high RNA quality. Consequently, it is improbable that the 5′ end bias is a direct consequence of the RNA extraction protocol. Finally, the fragments per kilobase million (FPKM) value was assessed across

the chromosome (Figure 7C). The number of transcripts mapped across the genome was evenly distributed, with up- and downward peaks across the profiles. These peaks are caused by the difference in up- and downregulated genes, to which more or less reads map, respectively.

Conclusion

We showed that enzymatic lysis through lysozyme treatment is an effective pretreatment for cell lysis of *N. europaea* cultures before RNA isolation with the silica-column based NucleoSpin RNA XS kit. Starting from only 5 mL of culture, we could extract more than 4 ng of high quality RNA from 10^6 cells. Therefore, in situations where the volume of low-biomass cultures is limited, the described protocol is able to deliver RNA samples with adequate yields. The final protocol was tested and validated on three strains: *N. europaea*, *N. winogradskyi*, and *C. testosteroni*. Furthermore, quality assessment of RNA-seq showed excellent quality for downstream processes. Hence, the RNA extraction procedure is effective to generate adequate RNA yields and quality from low and high biomass samples of different bacterial strains.

Limitations of the study

The developed procedure was validated on three bacterial strains. Lower yields are possible when working with different strains, less susceptible to lysozyme digestion. In addition, the enzymatic lysis pretreatment could also be effective in combination with other commercial silica spin column kits, not tested in this study.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105311.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.V. and F.M.; Methodology, T.V., R.V.H., and F.M.; Investigation, T.V., R.V.H., and F.M.; Writing – Original Draft, T.V. and F.M.; Writing – Review and Editing, all authors., Funding Acquisition, N.L., R.G., and F.M.; Resources: N.L., R.G., and F.M.; Supervision, N.L., R.G., and F.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Nitrosomonas europaea ATCC19718	AmericanType Culture Collection (ATCC)	NCBI: txid228410
Nitrobacter winogradskyi Nb-255	AmericanType Culture Collection (ATCC)	NCBI: txid323098
Comamonas testosteroni I2	Center for Microbial Ecology and Technology (CMET), UGhent	NCBI: txid1440775
Chemicals, peptides, and recombinant proteins		
Lysozyme from chicken egg-white	MedChemExpress	Cat#HY-B2237
Invitrogen™ Recombinant Proteinase K solution (20 mg/mL)	Invitrogen	Cat#AM2548
Ethylene diamine tetraacetic acid tetrasodium dihydrate	Sigma-Aldrich	Cat#E6511-100G
Trizma hydrochloride (TRIS-HCl)	Sigma-Aldrich	Cat#T3253
Critical commercial assays		
NucleoSpin RNA	Machery-Nagel	Cat#740955.50
NucleoSpin RNA XS	Machery-Nagel	Cat#740902.50
RNeasy Plus Mini (250)	QIAGEN	Cat#74136
RNeasy Plus Micro (50)	QIAGEN	Cat#74034
Direct-Zol™ RNA MiniPrep	Zymo Research Corp.	Cat#R2050
Agilent RNA 6000 Nano Kit	Agilent	Cat#5067-1511
Illumina Ribo-Zero Plus rRNA Depletion Kit	Illumina	Cat#20037135
Illumina TruSeq Stranded Total RNA	Illumina	Cat#20020597
Deposited data		
RNA-seq data of *Nitrosomonas europaea*	This paper; NCBI SRA Database	SRA: SRR21622767
RNA-seq data of *Nitrobacter winogradskyi*	This paper; NCBI SRA Database	SRA: SRR21622768
Software and algorithms		
Graphpad Prism v9.0.0	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
RStudio v1.4.1106	RStudio	https://www.rstudio.com/
Subread v2.0.1	Liao et al., 2013	http://subread.sourceforge.net/
RseQC v4.0.0	Wang et al., 2012	http://rseqc.sourceforge.net/
Qualimap v2.2.1	Okonechnikov et al., 2016	http://qualimap.conesalab.org/
Samtools v1.15.1	Danecek et al., 2021	http://www.htslib.org/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Felice Mastroleo (felice.mastroleo@sckcen.be).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- RNA-seq data has been deposited at the NCBI Sequence Read Archive (SRA) database and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Microbial strains

N. europaea ATCC 19718 was cultivated axenically in minimal medium (ATCC 2265) composed of 3.27 g/L $(NH_4)_2SO_4$, 5.83 g/L KH_2PO_4, 0.179 g/L $MgSO_4$ x $7H_2O$, 1.32e10^{-7} g/L $CuSO_4$ x $5H_2O$, 0.46 g/L NaH_2PO_4 and 0.397 Na_2CO_3 or in a synthetic urine salts solution (SUSS) medium at pH = 7.8, composed of 2.36 g/L $(NH_4)_2SO_4$, 0.15 g/L $NaNO_3$, 1.564 g/L KH_2PO_4, 2 g/L K_2HPO_4, 0.49 g/L $MgSO_4$ x $7H_2O$, 0.04 g/L $CaCl_2$ x $2H_2O$, 0.0014 g/L $FeSO_4$ x $7H_2O$, 5.2 g/L NaCl, 2.5 g/L $KHCO_3$, 3.2 g/L Na_2SO_4 x $10H_2O$ and 37.85 g/L EPPS buffer at a pH of 7.8. *N. winogradskyi* ATCC 25931 was grown axenically in SUSS medium where $(NH_4)_2SO_4$ was replaced by 2.46 g/L $NaNO_2$ as nitrogen source for *N. winogradskyi*, while pH was adjusted to 7.5. For both strains and all media types, cultures were subcultured by transferring 5% (v/v) of culture to fresh medium after 5–7 days of growth.

C. testosteroni I2 was grown in SUSS medium where 0.5 g/L Na-acetate was added as a C-source and where 1.07 g/L urea was added as N-source, replacing $(NH_4)_2SO_4$. pH was adjusted to 7.0. Subcultures were made by transferring 5% (v/v) of culture to fresh medium after 2 days of growth. All cultures were incubated at 30°C in the dark on an orbital shaker shaking at 120 rpm in 50 mL red cap CELLSTAR® cell culture flasks (Greiner Bio-One, Kremsmünster, Austria).

METHOD DETAILS

Sample preparation

The optical density (OD_{600}) was determined with a NanoColor UV/VIS II spectrophotometer (Machery-Nagel Inc., Allentown, PA, USA) and samples were harvested after 5–7 days for *N. europaea* and *N. winogradskyi* and after 2 days for *C. testosteroni*. 5 mL of culture was harvested by centrifugation (16,000 g, 5 min at 4°C).

For *N. europaea* and *N. winogradskyi* cultures, cell counts were calculated based on linear regression of the measured OD_{600} with cell count as previously reported (Farges et al., 2012):

$$\frac{N.\ europaea}{mL} = 1.010 \times 10^9 \times OD_{600} \pm 0.022 \times 10^9 \quad \text{(Equation 1)}$$

$$\frac{N.\ winogradskyi}{mL} = 1.779 \times 10^9 \times OD_{600} \pm 0.091 \times 10^9 \quad \text{(Equation 2)}$$

RNA extraction

RNA extractions were performed immediately after sample harvesting. Five Silica-column based extraction kits from three different companies were tested (as listed in the key resources table).

Manufacturer's instructions were followed unless stated otherwise The DZ kit does not provide a lysis buffer but recommends the use of any acid-guanidine-phenol reagent for cell lysis. In this research, TRIzol® (Thermo Fischer Scientific, Walthan, MA, USA) was used. Total elution volume was a result of applying half the elution volume on the column twice. The RNA concentration and quality (RIN) was determined with a 6000 Nano Lab-Chip kit and the Agilent BioAnalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA). By default, the BioAnalyzer can calculate a RIN-value if the RNA concentration of the sample is greater than 10 ng/μL. Every RNA extraction procedure was performed on at least 3 biological replicates. Total RNA yield was determined by multiplying the measured RNA concentration (ng/μL) with the total elution volume. The total RNA yield was normalized to the cell count and expressed in ng RNA $\times$ 10^{-6} cells, where total cell count of the samples was calculated using formula (1) and (2) multiplied by the volume for total cell count.

The effect of additional lysis steps and modifications to the standard kit protocol of the NucleoSpin RNA XS extraction kit was also investigated. These include the addition of mechanical lysis by ultrasonication and enzymatic lysis with a lysozyme solution and/or proteinase K at different concentrations and with different incubation times. Moreover, adjustments were made to the standard procedure where specified. All different procedures were compared based on their generated RNA yield and the quality of the extracted RNA.

In mechanical lysis procedures, cells were lysed using an ultrasonication probe (UP50H Ultrasonic Processor, Hielscher Ultrasonics, Teltow, Germany). First, harvested pellets were resuspended in lysis buffer provided with the commercial kit that was used. Then, the samples were ultrasonicated at different intensities and/or over different time intervals and were put on ice between cycles of ultrasonication treatment.

In enzymatic lysis procedures, samples were resuspended in varying concentrations of lysozyme from chicken egg white (MedChemExpress, Monmouth Junction, NJ, USA) dissolved in 200 μL 1x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8.00). Where stated, they were incubated at 37°C for 15 min with a Mixer HC (STARLAB, Milton Keynes, UK) heating block either static or shaking at 1,400 rpm. A 20 mg/mL proteinase K solution (Invitrogen™, Waltham, MA, USA) was added to the lysozyme solution where specified.

Library construction and RNA sequencing and read mapping

RNA sequencing procedure was outsourced to BaseClear B.V. (Leiden, The Netherlands). BaseClear B.V. used an input of 200 ng of RNA. Then, rRNA was first depleted using the Illumina Ribo-Zero Plus rRNA depletion kit. The Illumina TruSeq Stranded Total RNA kit was used to construct the library. Paired-end sequence reads were generated using the Illumina NovaSeq 6000 system. The sequences generated with the NovaSeq 6000 were performed under accreditation according to the scope of BaseClear B.V. (L457; NEN-EN-ISO/IEC 17025). FASTQ read sequence files were generated using bcl2fastq version 2.20 (Illumina). Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing PhiX control signal were removed using an in-house filtering protocol. In addition, reads containing (partial) adapters were clipped (up to a minimum read length of 50 bp). The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.11.8.

Read mapping and quality control

Paired-end reads were aligned with subread (version 2.0.1). (Liao et al., 2013) to the respective reference genomes of the strain from which the sample originated (*N. europaea* ATCC, 19718; NCBI accession number AL954747.1, *N. winogradskyi* Nb-255; NCBI accession number CP000115.1). The resulting bam files were sorted and indexed with Samtools (version 1.15.1) (Danecek et al., 2021). RNA quality aspects (read coverage over gene body, Transcript Integrity Number(TIN) values, Fragments per Kilobase Million (FPKM) counts) were analyzed with RSeQC (version 4.0.0) (Wang et al., 2012) and Qualimap v.2.2.1 (Okonechnikov et al., 2016).

QUANTIFICATION AND STATISTICAL ANALYSIS

For data shown in Figures 1, 3, 4, and 6, Shapiro-Wilk test to test for normality, ordinary one-way Analysis of Variance (ANOVA) and post-hoc pairwise Tukey-tests were used. For data shown in Figure 2, normality was checked with Shapiro-Wilk test and a two-sided Student's *t* test statistic was used for statistical analysis. All statistical tests were performed using Graphpad Prism version 9.2.0 for Windows (Graphpad Software, San Diego, CA, USA). Significance was defined when a statistical test provided a p value < 0.05. Statistical details are provided in the figure legends. Error bars represent the standard deviation from the mean. In all cases, data was obtained from at least 3 biological replicates.