AsChip: A High-Throughput qPCR Chip for Comprehensive Profiling of Genes Linked to Microbial Cycling of Arsenic

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Supporting Information

ABSTRACT: Arsenic (As) is a ubiquitous toxic element adversely affecting human health. Microbe-mediated cycling of As is largely mediated by detoxification and energy metabolism in microorganisms. We here report the development of a novel high-throughput qPCR (HT-qPCR) chip (AsChip) for comprehensive profiling of genes involved in microbial As cycling (here collectively termed “As genes”). AsChip contained 81 primer sets targeting 19 As genes and the 16S rRNA gene as a reference gene. Gene amplicon sequencing showed high identity (>96%) of newly designed primers corresponding to their targets. AsChip displayed high sensitivity (plasmid template serial dilution test; r = −0.99), with more than 96% of all PCR assays yielding true positive signals. R² coefficients for standard curves and PCR amplification efficiencies averaged 0.98 and 0.99, respectively. A high correlation between C_T values obtained by AsChip and conventional qPCR was obtained (r = 0.962, P < 0.001). Finally, we successfully applied AsChip on soil samples from a chromium−copper−arsenic-contaminated field site and identified diverse As genes with total abundance averaging 0.4 As gene copies per 16S rRNA. Our results indicate that AsChip constitutes a robust tool for comprehensive quantitative profiling of As genes in environmental samples.

INTRODUCTION

Arsenic (As) is a ubiquitous toxic element impacting human health. It is present in the atmosphere, hydrosphere, pedosphere, and biosphere with both natural and anthropogenic origins. Arsenic in the environment primarily poses health risks to humans via water or food and has been linked to various cancers, cardiovascular disease, diabetes, and skin lesions. The toxicity, mobility, and environmental fate of As are to a large extent determined by its chemical speciation. For example, inorganic As species prevalent in drinking water are much more toxic than arsenobetaine, which is the primary source of As in fish meat. Hence, the following toxicity order of arsenicals have been reported for humans: inorganic As(III) > dimethylarsenite [DMAs(III)] > dimethylarsenate [DMAs(V)] > monomethylarsonate [MMAs(V)] > inorganic As(V).

Environmental As speciation is profoundly impacted by microbial activity. Dissimilatory As oxygenation and reduction constitute ancient types of bacterial energy metabolism, whereas other As biotransformation processes can be linked to As detoxification processes conferring bacterial resistance to As. Collectively, microorganisms (mainly prokaryotes) transforming As can be divided into several more or less distinct functional groups, such as chemoautotrophic arsenite-oxidizing bacteria (CAO) generating energy from the dissimilatory oxidation of arsenite with O₂ or nitrate as terminal electron acceptor, dissimilatory arsenate-reducing anaerobic prokaryotes (DARP) generating energy from the oxidation of various organic or inorganic electron donors with arsenate as the terminal electron acceptor, and prokaryotes transforming As compounds for other physiological purposes (e.g., As resistance/detoxification). Where the two first mentioned groups consist of specialized prokaryotes

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generating energy from inorganic As biotransformations, the latter group includes prokaryotes carrying out a wide range of contrasting As biotransformation processes. A major subgroup includes heterotrophic arsenite oxidizers (HAO) transforming toxic arsenite into the less toxic arsenate. Conversely, other bacteria reduce arsenate into arsenite and couple it to arsenite efflux to confer As resistance. Finally, a range of microorganisms transform organoarsenicals, of which many are volatile and highly mobile. Despite their immense biogeochemical significance, only little is known about the physiological purposes of these As biotransformations.2

A large number of genes (here collectively referred to as “As genes”) are involved in microbe-mediated cycling of As and constitute promising functional gene markers for studies of As biotransformation processes and the various functional groups of prokaryotes that carry out these processes.18 For example, inorganic arsenate can be reduced to arsenite by arsenate reductase encoded by \( \text{arsC} \) (resistance) or \( \text{arrAB} \) (anaerobic As respiration), whereas the toxic As(III)/MAs(III) can be pumped out of bacterial cells by arsenic transporters encoded by \( \text{arsB} \), \( \text{arsP} \), or \( \text{acr3} \) (resistance). Arsenite or organoarsenical oxidases encoded by \( \text{arsA} \) (dissimilatory arsenite oxidation), \( \text{aioAB} \) (resistance), or \( \text{arsH} \) (resistance) may further transform As(III)/MAs(III) to the less toxic As(V)/MAs(V). As methylation is encoded by \( \text{arsM} \) (resistance) and results in the loss of As by volatilization or products such as mono-, di- or trimethylarsonines.21 Demethylation of trivalent organoarsenicals (MAs[III] and roxarsone) is catalyzed by C-As lyase encoded by \( \text{arsI} \) as part of a resistance pathway.22–24

Our knowledge about As biotransformation processes and the underlying microbial ecology is incomplete in part due to the lack of comprehensive, targeted methods to comprehensively study As-transforming microorganisms. With the development of next-generation sequencing (NGS) technologies and their decreasing costs, metagenomic and metatranscriptomic studies of As biotransformation are beginning to emerge.25–27 However, these approaches only provide relative abundances of targeted genes and their transcripts and should therefore ideally be complemented by their high throughput quantification. Quantitative polymerase chain reaction (qPCR) represents one of the most widely applied techniques for quantifying functional genes and has already been used in several environmental studies of As biogeochemistry.21,28–37 This technique generally provides more sensitive detection of genes in environmental samples as compared to hybridization microarray technology (e.g., the GeoChip).38,39 However, conventional single-gene qPCR gets laborious and impractical when many genes have to be quantified at the same time as would be necessary for comprehensive monitoring of all As genes in environmental samples.35,37 Hence, we aimed to develop and validate a novel high-throughput qPCR (HT-qPCR)-based chip for sensitive, rapid, and cost-efficient quantification of known prokaryotic As genes. The developed HT-qPCR chip (AsChip) targets 19 microbe-mediated As cycling genes and the bacterial 16S rRNA gene as a reference gene. AsChip employs a total of 81 cross-species primer sets covering hundreds of species with 70 primer sets being newly developed and validated as part of the present study.
Table 1. Summary of Genes Targeted by AsChip, Microorganisms Hosting Genes, and Numbers of AsChip qPCR Primers Used To Detect Genes*

<table>
<thead>
<tr>
<th>Gene type</th>
<th>Functional process</th>
<th>Encoded proteins</th>
<th>Functionality</th>
<th>Microbes</th>
<th>No. of primer pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>aoxA/aoxB</td>
<td>As(III) oxidation</td>
<td>As(III) oxidase small subunit</td>
<td>As(III) oxidation</td>
<td>HAO</td>
<td>3 1 73</td>
</tr>
<tr>
<td>aoxR/aoxR</td>
<td>As(III) oxidation</td>
<td>As(III) oxidase large subunit</td>
<td>As(III) oxidation</td>
<td>HAO</td>
<td>3 1 73</td>
</tr>
<tr>
<td>aoxS/aoxS</td>
<td>response regulator</td>
<td>regulation of aox/aox operon expression</td>
<td>HAO</td>
<td>3 1 73</td>
<td></td>
</tr>
<tr>
<td>arsH</td>
<td>organoarsenical oxidase</td>
<td>trivalent organoarsenicals oxidation</td>
<td>ARM</td>
<td>3 0 23</td>
<td></td>
</tr>
<tr>
<td>aoxC/aoxC</td>
<td>cytochrome c</td>
<td>encoding c-type cytochrome in aox/aox operon</td>
<td>HAO</td>
<td>1 1 74</td>
<td></td>
</tr>
<tr>
<td>aoxD/aoxD</td>
<td>chlE - molybdoenoptein biosynthesis</td>
<td>encoding an enzyme involved in molybdoenoptein biosynthesis in aox/aox operon</td>
<td>HAO</td>
<td>1 1 74</td>
<td></td>
</tr>
<tr>
<td>arsA</td>
<td>As(III) oxidase</td>
<td>As(III) oxidation</td>
<td>CAO</td>
<td>1 0 75</td>
<td></td>
</tr>
<tr>
<td>arrA</td>
<td>As(V) reduction</td>
<td>As(V) oxidase</td>
<td>DARP</td>
<td>1 1 36, 76</td>
<td></td>
</tr>
<tr>
<td>arrB</td>
<td>As(V) respiratory reductase large subunit</td>
<td>As(V) reduction</td>
<td>DARP</td>
<td>1 0 36, 77</td>
<td></td>
</tr>
<tr>
<td>arrC</td>
<td>As(V) reductase</td>
<td>As(V) reduction</td>
<td>ARM</td>
<td>13 2 77</td>
<td></td>
</tr>
<tr>
<td>arrR</td>
<td>transcriptional repressor</td>
<td>regulation of arr operon expression</td>
<td>ARM</td>
<td>8 0 77</td>
<td></td>
</tr>
<tr>
<td>arsI</td>
<td>Arsenic methylation and demethylation</td>
<td>C–As bond lyase</td>
<td>demethylation of trivalent organoarsenicals to less toxic As(III)</td>
<td>AMM</td>
<td>4 0 23</td>
</tr>
<tr>
<td>arsM</td>
<td>As(III) methyltransferases</td>
<td>arsenic methylation</td>
<td>AMM</td>
<td>5 0 23</td>
<td></td>
</tr>
<tr>
<td>aorA</td>
<td>Arsenic transport</td>
<td>As(III)-activated ATPase</td>
<td>catalysis of oxyanion-translocating ATPase</td>
<td>ARM</td>
<td>7 0 78</td>
</tr>
<tr>
<td>aorB</td>
<td>As(III) efflux pump protein</td>
<td>extrusion of As(III) from the cell</td>
<td>ARM</td>
<td>6 0 79</td>
<td></td>
</tr>
<tr>
<td>aorD</td>
<td>arsenical metallochaperone</td>
<td>transfer of trivalent metalloids to aorA</td>
<td>ARM</td>
<td>3 0 78</td>
<td></td>
</tr>
<tr>
<td>aorP</td>
<td>trivalent organoarsenicals permease</td>
<td>extrusion of trivalent organoarsenicals from the cell</td>
<td>ARM</td>
<td>2 0 80</td>
<td></td>
</tr>
<tr>
<td>aorC</td>
<td>As(III) efflux pump protein</td>
<td>extrusion of As(III) from the cell</td>
<td>ARM</td>
<td>2 1 81, 82</td>
<td></td>
</tr>
<tr>
<td>Total 19 genes</td>
<td></td>
<td></td>
<td></td>
<td>70 10</td>
<td></td>
</tr>
</tbody>
</table>

*Detailed information on all primer sets is shown in Table S1. Abbreviations used: Heterotrophic arsenite oxidizers, HAO; Arsenic resistant microbes, ARM; Chemoautotrophic arsenite oxidizers, CAO; Dissimilatory arsenate-reducing prokaryotes, DARP; Arsenic methylating microbes, AMM.

MATERIALS AND METHODS

Design of AsChip. Major steps for AsChip design are shown in Figure 1a. A total of 19 targeted genes of relevance for microbial cycling of As were selected (Table 1). Collectively, these targeted genes encode As(III) oxidation, As(V) reduction, As methylation, As demethylation, and As transport (efflux). Ten validated primer pairs were selected from previously published studies, whereas a total of 70 novel primer pairs were designed as part of this study (Table 1; Table S1). Primer design was performed based on target gene sequences retrieved from the National Center for Biotechnology Information (NCBI) GenBank database. Gene sequences were obtained from both bacteria and archaea and were further collected and aligned using MEGA 7.0 with ClustaW. Sequences were clustered into different groups using a neighbor-joining phylogeny tree based on alignment results. SYBR Green Design of AlleleID 6.0 was used to design taxa-specific/cross-species primers for top clustered groups of each gene. The cross-species primers were designed based on conserved regions found in clustered groups. The quality of designed primers and expected priming efficiency were further checked by AlleleID 6.0 with the following criteria: primer length of 18–24 bases, amplicon length of 50–200 bases, GC content of 40%–60%, expected melting temperature (Tm) of product at 48–62 °C, hairpin ΔG ≥ −3 kcal/mol, self-dimer ΔG ≥ −6 kcal/mol, cross-dimer ΔG ≥ −6 kcal/mol, and GC clamp ≤3.40,41 Only primers meeting the above quality criteria were used for further validation and application. The bacterial 16S rRNA gene was included in the construction of AsChip as a reference gene using the universal primers 1108F/1132R.42 Detailed information on the 81 primer sets is shown in Table S1.

Validation of AsChip. Major steps of AsChip validation are shown in Figure 1b. The specificity of AsChip was validated computationally and experimentally. Computationally, Primer-BLAST43 was used to check newly designed primers for specificity against the NCBI nucleotide collection (nr) database with bacteria (taxid:2) and archaea(taxid:2157) as target organisms with a max target size of 1000 bases. All newly designed primers were also tested experimentally by PCR using DNA extracted from contrasting environmental samples as templates. DNA were extracted from samples...
collected from a chromium–copper–arsenic contaminated soil, estuaries (sediment), and urban sewage (water). Water samples were filtered through a 0.22 μm cellulose nitrate membrane filter to collect bacterial pellets prior to DNA extraction. Detailed protocols for DNA extraction of sediment and water samples were described previously. The detailed protocol for DNA extraction from soil samples is described in the section entitled Application of AsChip on Environmental Samples. The purity of DNA was checked (ND-1000, Nanodrop, USA), and DNA was diluted to the same concentration with sterile water for later use. A total of 53 different PCR amplicons were detected and checked via agarose gel electrophoresis and Sanger sequencing (company Bioray, Xiamen, China). Amplicon sequences were assembled and aligned with target sequences using the software DNAMAN v9.0. Furthermore, AsChip primer specificities were routinely monitored by analysis of melting curves in all downstream AsChip HT-qPCR experiments using the Wafergen SmartChip Real-time PCR system. For regular PCR, each 50 μL PCR reaction contained 25 μL TaKaRa PremixTaq (ExTaq), 1 μL of each primer, 22 μL PCR grade water, and 1 μL DNA template. The thermal cycle consisted of an initial polymerase activation at 95 °C for 10 min and 40 cycles of denaturation (95 °C, 30s), annealing (58 °C, 30s), and extension (72 °C, 120s).

The serial dilution test and standard curve analysis were conducted to validate specificity, sensitivity, and efficiency using plasmids as positive controls. Gene fragments targeted by all the 70 newly designed primers were synthesized and cloned into plasmids cloning vector pUC57-Ampicillin (GENEWIZ, Plainsboro, NJ, USA). Amplicon sequences were assembled and aligned with target sequences using the software DNAMAN v9.0. Furthermore, AsChip primer specificities were routinely monitored by analysis of melting curves in all downstream AsChip HT-qPCR experiments using the Wafergen SmartChip Real-time PCR system. For regular PCR, each 50 μL PCR reaction contained 25 μL TaKaRa PremixTaq (ExTaq), 1 μL of each primer, 22 μL PCR grade water, and 1 μL DNA template. The thermal cycle consisted of an initial polymerase activation at 95 °C for 10 min and 40 cycles of denaturation (95 °C, 30s), annealing (58 °C, 30s), and extension (72 °C, 120s).

The thermal cycle consisted of initial enzyme activation for 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30s, annealing at 58 °C for 30s, and extension at 72 °C for 120s.

The qPCR primer amplification efficiency was calculated as As gene copy numbers per 16S rRNA gene copy number (Relative As gene copy number) normalized As gene abundances were calculated as: normalized As gene copy number = (relative As gene copy number/relative 16S rRNA gene copy number) × absolute 16S rRNA gene copy number, where relative gene copy number = 10^[(log_{10} C_T) - (log_{10} C_{T})]/(T_m)] of the product generated by AlleleID also considered. The thermal cycle consisted of initial enzyme activation 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30s, annealing at 48 to 60 °C with

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Results and Discussion

Coverage and Specificity of AsChip. The developed AsChip targets almost all known prokaryotic As genes (Table 1 and Table S1). Validations of AsChip primer specificities were performed both in silico using Primer-BLAST and experimentally. Primer-BLAST identified a total of approximately 6500 target sequences with the 70 newly designed primers. Almost all gene targets (98.9%) were derived from diverse bacterial phyla, whereas the remaining fraction originated from archaea (Euryarchaeota). Bacterial phyla
covered by AsChip primers included Proteobacteria, Firmicutes, Actinobacteria, Bacteriodetes, Cyanobacteria, Verrucomicrobia, and Fusobacteria. We randomly selected five As genes in order to calculate the fraction of known annotated As gene sequences (Genbank) that could be amplified by the designed AsChip primers. The fraction of sequences targeted by the selected As genes were as follows: *aoxA* (67%; 4 out of 6), *arsC* (80%; 228 out of 286), *arsH* (60%; 116 out of 192), *arsM* (62%; 46 out 74), and *arsR* (64%; 492 out of 772), demonstrating adequate coverage of AsChip. Primer-BLAST matches of 100% between primers and their target sequences were obtained for all designed primers using the NCBI nr database (organisms limited to Bacteria and Archaea). Primer specificities were further tested experimentally by the melting curve analysis on HT-qPCR reactions with all 70 newly designed primers targeting As genes. All melting curves of qPCR generated with AsChip showed the presence of a single peak, suggesting the success of designed primers for their specificity. The specificity was double checked and further confirmed by Sanger sequencing of all amplified PCR products (*n* = 53) obtained with newly designed primer pairs. Agarose gel electrophoresis of amplified PCR products in all cases showed a single band of the expected size, and DNA sequencing further revealed that the obtained amplicons corresponded well to their respective targets (Figure 2). Hence, amplicon sequences in all cases showed high similarity to their targets (>96%) with 89% of the primers resulting in amplicon sequences with ≥99% similarity to their respective targets. The serial dilution test displayed true-positive amplifications of only targeted sequences, whereas no amplicons could be detected for negative controls (i.e., no template) with the absence of any primer secondary structures. Collectively, our results thus demonstrated a high and satisfactory specificity of AsChip suggesting a low risk for false positive detection of As genes.

**Sensitivity and Efficiency of HT-qPCR AsChip and Comparison with Conventional qPCR.** The performance of AsChip was assessed by estimating its sensitivity using standard curves generated by serial dilution test with mixed plasmids serving as positive controls (Figure 3; Figure S1). The results demonstrated a high sensitivity of AsChip. *C*₇ values corresponding to positive plasmid template concentrations of 4 × 10⁻⁵ to 4 ng μL⁻¹ ranged from 7 to 31. Within this template concentration range, more than 96% of all PCR assays yielded true positive signals, whereas standard curve Pearson correlation coefficients and *R*² (4 × 10⁻⁵ to 4 ng μL⁻¹ range) averaged −0.99 and 0.98, respectively (Table S2). For the more efficient application of AsChip, the optimal cutoff point, the LoD, and the limit of quantification (LoQ) were determined by standard curve analysis of serial dilution (Table S3). The optimal cutoff points for *C*₇ values of the 70 newly designed primers ranged from 26 to 31 with an average of 30. The LoD varied from 7 to 262 target copies with 61 as the average, while LoQ varied from 11 to 132,667 target copies with an average of 10,319.

HT-qPCR AsChip performance for all 70 newly designed primers was further validated by calculating qPCR efficiency indices and *R*² based on standard curves (Figure 4; Table S2). PCR efficiency indices averaged 0.99 with a standard deviation of 0.093.

![Figure 2](image2.png)

**Figure 2.** Identity of primers to their targets (%). The figure depicts the distribution of PCR assays (%) against the amplicon sequence similarity (%) to their respective targets used for primer design.

![Figure 3](image3.png)

**Figure 3.** AsChip sensitivity (serial dilution test) with 70 newly designed primers as evaluated by 10-fold serial dilution of mixed positive plasmid controls. Dots on the right side of boxes represent data obtained for individual primer pairs. Plasmid DNA concentrations are indicated in the figure (upper right). IQR, interquartile range.

![Figure 4](image4.png)

**Figure 4.** Distribution of AsChip HT-qPCR amplification efficiency with 70 newly designed primers. Standard curves and their parameters are shown in Figure S1 and Table S2.
of 0.09 and ranged between 0.77 and 1.26 with the vast majority in the range of 0.8 − 1.2 (Figure 4). $R^2$ coefficients for standard curves averaged 0.98 ± 0.01 (±standard deviation). A potential deviation on normalized abundances (i.e., As gene copy number per 16S rRNA gene) might occur when applying comparative $C_T$ since it was based on the assumption of the same amplification efficiency of all amplicons. However, for future AsChip application, this can be overcome with absolute quantification of each target with the standard curve method, by increasing primer concentration, or by optimizing the primer to specific $T_m$.

The high sensitivity and robust performance of AsChip were further confirmed by comparing $C_T$ values obtained by nanoliter-scale AsChip analysis with traditional microliter-scale qPCR (Figure 5). $C_T$ values obtained by the two qPCR platforms showed a significant positive correlation (Pearson’s $r = 0.962, P < 0.001$), indicating a high degree of reliability and reproducibility of AsChip (Figure 5). Hence, similar PCR efficiencies and detection limits were obtained by the two qPCR platforms in accordance with previous reports from the development of other HT-qPCR chips. A total of 52 As gene targets could be detected by both conventional qPCR and AsChip, whereas an additional seven As genes could only be detected by AsChip suggesting a higher sensitivity of AsChip. Other possible explanations for the observed slight differences between results obtained with the two qPCR platforms include automated versus manual qPCR reagent handling, laboratory contaminations, and inaccuracies of DNA quality quantified by UV absorbance.

**Application of AsChip.** AsChip analysis was carried out on DNA extracted from three Danish top soils collected from a site contaminated with different levels of chromium, copper, and arsenic. The three soils were labeled “low”, “medium”, or “high”, depending on the arsenic contamination level (33, 203, or 2839 $\mu$g A$g^{-1}$, respectively). The absolute abundances of As genes (copies per gram of soil) ranged from $8.28 \times 10^9$ to $1.34 \times 10^{11}$ (average of $7.30 \times 10^{10}$; Table S4), but total normalized abundances varied much less and were 0.43, 0.51, and 0.32 in low, medium, and high contaminated soils. Hence, our data indicate that the overall As gene abundance did not vary much with soil As contamination level.

The normalized abundance of individual As genes are shown in Figure 6. Out of 19 different As genes, 17 could be detected in the three soils demonstrating high potential for microbial As transformations. Remarkably, the arxA gene encoding the arsenite oxidase in chemoautotrophic arsenite-oxidizing bacteria (CAO) was not detected in any of the three contaminated soils suggesting that these specialized bacteria generating energy from the oxidation of arsenite with molecular oxygen or nitrate as terminal electron acceptor were not active in the studied soils. This finding is consistent with arsenate being the dominant inorganic As species and the

![Figure 5](image-url)  
**Figure 5.** AsChip performance as evaluated against conventional single-gene qPCR by comparison of $C_T$ (cycle threshold) values ($n = 52$). Linear regression analysis revealed a linear correlation (Pearson’s $r = 0.962, P < 0.001$) between the $C_T$ values obtained from the two qPCR platforms (conventional single-gene qPCR and AsChip).

![Figure 6](image-url)  
**Figure 6.** Log$_{10}$ transformed normalized As gene abundance (copy number per 16S rRNA) of 19 genes of relevance for microbe-mediated biogeochemical cycling of As in three top soils. Soils were collected from a chromium–copper–arsenic-contaminated site and named “Low” (33 $\mu$g A$g^{-1}$), “Medium” (203 $\mu$g A$g^{-1}$), or “High” (2839 $\mu$g A$g^{-1}$). Samples were clustered based on Pearson distance using hierarchical algorithms (average linkage cluster). Abbreviations used: HAO, heterotrophic arsenite oxidizers; ARM, arsenic resistant microorganisms; CAO, chemoautotrophic arsenite oxidizers; DARP, dissimilatory arsenate-reducing prokaryotes.
anticipated absence of an As redox stratification in the studied upland soils.

Two of the most abundant As genes were \textit{arsB} (0.049–0.069 copies per 16S rRNA gene) and \textit{arsC} (0.18–0.30 copies per 16S rRNA gene). Collectively, these genes encode arsenate resistance by the arsenate-reduction-arsenate efflux pathway. Hence, our AsChip data indicates that the \textit{arsBC} encoded resistance mechanism is very common in bacteria irrespective of the As contamination level of their natural habitats. This is consistent with the frequent occurrence of \textit{arsBC} in cultured bacteria (e.g., \textit{Escherichia coli}) isolated from noncontaminated habitats. Interestingly, AsChip also consistently detected a series of As genes (\textit{arsH}, \textit{arsI}, and \textit{arsM}) involved in recently discovered parallel pathways for organoarsenical biotransformation and detoxification. The \textit{arsM} gene encodes a \textit{As}(III) S-adenosylmethionine methyltransferase that transforms inorganic arsenite into the even more toxic organoarsenical methyl arsenite (a human carcinogen) which can be rapidly oxidized to its less toxic pentavalent form, whereas \textit{arsI} and \textit{arsH} encode different methyl arsenite detoxification mechanisms. Other arsenic-resistant genes (\textit{arsADRP}, \textit{acr3}) were also detected in our samples demonstrating the existence of several As resistance pathways in the studied microbial communities.

\textit{AoxABCRS} genes (also called \textit{aioABCRS}) are involved in heterotrophic arsenite oxidation and were detected in all samples with the \textit{aox} gene being the most abundant of the \textit{aox} genes and the second most abundant As gene overall (0.04, 0.11, and 0.07 copies per 16S rRNA gene in low, medium, and high contaminated soil, respectively). The \textit{aoxB} gene (large Mo-protein subunit of \textit{As}(III) oxidase at 7.1 × 10^6, 2.8 × 10^6, and 1.7 × 10^6 copies g^{-1}, correspondingly) was 10, 45 and 26 folds more abundant than the \textit{aoxA} gene (small Fe–S Rieske subunit of \textit{As}(III) oxidase, 5.7 × 10^6, 6.3 × 10^6, and 6.7 × 10^6 copies g^{-1}, correspondingly) in the soils named low, medium, and high, respectively (Table S4). This was not due to differences in PCR amplification efficiencies and could thus most likely be attributed to lower coverage of the primers used for \textit{aoxA} as compared to \textit{aoxB}. This interpretation is consistent with \textit{aoxB} (also named \textit{aioA}) being more frequently studied as compared to \textit{aoxA} (also named \textit{aioB}), and more target sequences were thus available in databases for \textit{aoxB} primer design. Nevertheless, differences in the normalized abundance for the three studied soils followed the same order for both \textit{aoxA} and \textit{aoxB} suggesting that the current first-generation AsChip can still be used to monitor relative differences between samples. In contrast to the \textit{aoxAB} gene pair, the genes \textit{arrA} and \textit{arrB} encoding the large and the small subunit of \textit{As}(V) reductase in dissimilatory arsenate-reducing prokaryotes (DARPs) were found in near equal abundances for each soil, suggesting that AsChip primers used for detection of \textit{arrA} and \textit{arrB} displayed similar bacterial community coverage. Hence, \textit{arrA} gene copy numbers were 6.2 × 10^6, 2.4 × 10^6, and 2.1 × 10^6 in soil named low, medium, and high, whereas corresponding numbers for \textit{arrB} were 10.0 × 10^6, 2.9 × 10^6, and 1.2 × 10^6 copies g^{-1}, respectively (Table S4).

To our knowledge, AsChip represents the first HT-qPCR-based chip for As genes, and our results indicate that it can be used as a robust and reliable tool for comprehensive quantitative profiling of As genes in environmental samples. Hence, AsChip provides specific, sensitive, and targeted quantification of As genes and may be used in various environments for better understanding of As microbe-mediated biotransformations and its biogeochemical cycling. The high sensitivity and PCR reaction miniaturization allow AsChip analysis to be performed with only a small amount of template DNA. Furthermore, HT-qPCR-based chips have already been successfully developed and applied for other genes in various environments. Consequently, HT-qPCR data analysis is already well developed and allows researchers to perform absolute quantification, relative quantification, or comparative quantification of the targeted genes. Moreover, results obtained from AsChip may potentially be used to link detected As genes to their putative microbial hosts via the Primer-BLAST tool. The microarray-based GeoChip also includes probes targeting As genes and has been used in a previous study of As biotransformation processes. However, the GeoChip was designed for a wide range of functional genes and not specifically for As genes, and only a limited diversity of As genes were targeted (i.e., \textit{arsABCM} and \textit{aoxB}). Compared with GeoChip, AsChip presented in this study thus displays better As gene coverage and, most likely, higher sensitivity for As gene quantification.

Although the current first-generation AsChip has great potential as a comprehensive molecular tool for studies of microbial As cycling, novel functional groups of As-biotransforming prokaryotes and As genes not matching the current AsChip primers are likely to be discovered and characterized in the future. This implies that AsChip will have to be further refined and improved. The literature already contains reports of As genes that were not included in AsChip design. For instance, two novel arsenic resistance genes \textit{arsO} (for a putative flavin-binding monooxygenase) and \textit{arsT} (for a putative thiorodoxin reductase) were reported in a previous study. Likewise, the As resistance genes \textit{arsN} (encoding acetyltransferase), \textit{arsJ} (encoding organoarsenical efflux permease), and \textit{getB} (encoding glutathione S-transferase B) were reported previously, but only limited coding sequence information is currently available for primer design. Despite these limitations, AsChip offers unprecedented potential for targeted, yet comprehensive, quantification of As genes in environmental samples. We propose that AsChip may be applied together with other “omics” methods and (geo)chemical analysis in future studies of As resistance, biotransformation, and biogeochemistry. Such polyphasic approaches may reconcile geochemical and genomics data for the development of systems models to predict the fate and impacts of As and the movement of microorganisms in the environment.
The authors declare no competing financial interest.

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