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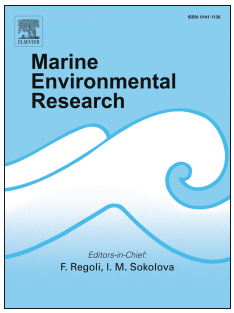

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Microbial strains isolated from CO_2-venting Kolumbo submarine volcano show enhanced co-tolerance to acidity and antibiotics

Manolis Mandalakis[a,*], Asimenia Gavriilidou[a,1], Paraskevi N. Polymenakou[a], Christos A. Christakis[a], Paraskevi Nomikou[b], Matej Medvecký[c,d], Stephanos P. Kilias[b], Maroudio Kentouri[e], Georgios Kotoulas[a] and Antonios Magoulas[a]

[a]Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, 71500 Heraklion, Greece

[b]Faculty of Geology and Geoenvironment, National and Kapodistrian University of Athens, Panepistimioupoli Zographou, 15784 Athens, Greece

[c]National Centre for Biomolecular Research, Faculty of Science, Masaryk University, 61137 Brno, Czech Republic

[d]Veterinary Research Institute, 62100 Brno, Czech Republic

[e]Department of Biology, University of Crete, 70013 Heraklion, Greece

[1]Present address: Laboratory of Microbiology, Wageningen University & Research, 6703 HB Wageningen, Netherlands

[*] Corresponding author, E-mail address: mandalakis@hcmr.gr, phone: +30-2810-337855, fax: +30-2810-337822 (M. Mandalakis).

Abstract

As ocean acidification intensifies, there is growing global concern about the impacts that future pH levels are likely to have on marine life and ecosystems. By analogy, a steep decrease of seawater pH with depth is encountered inside the Kolumbo submarine volcano (northeast Santorini) as a result of natural CO_2 venting, making this system ideal for ocean acidification research. Here, we investigated whether the increase of acidity towards deeper layers of Kolumbo crater had any effect on relevant phenotypic traits of bacterial isolates. A total of 31 *Pseudomonas* strains were isolated from both surface- (SSL) and deep-seawater layers (DSL), with the latter presenting a significantly higher acid tolerance. In particular, the DSL strains were able to cope with H^+ levels that were 18 times higher. Similarly, the DSL isolates exhibited a significantly higher tolerance than SSL strains against six commonly used antibiotics and As(III). More importantly, a significant positive correlation was revealed between antibiotics and acid tolerance across the entire set of SSL and DSL isolates. Our findings imply that *Pseudomonas* species with higher resilience to antibiotics could be favored by the prospect of acidifying oceans. Further studies are required to determine if this feature is universal across marine bacteria and to assess potential ecological impacts.

Keywords: Submarine volcanoes; Extreme environments; Bacteria; *Pseudomonas*; Acidification; Antibiotic/acid tolerance; Heavy metals; Marine microbial ecology

1. Introduction

Based on historical global estimates, human activities (i.e. fossil fuel combustion, cement production and deforestation) have caused the emission of around 555 PgC carbon dioxide between 1750 and 2011 (Ciais et al., 2013). With almost half of the emissions being accumulated in the atmosphere, the ambient levels of CO_2 increased from 278 to 390 ppm over the same time period (Ciais et al., 2013). Accordingly, the oceans are estimated to have absorbed about 28% of cumulative anthropogenic CO_2 emissions, which has resulted in the decrease of global mean surface ocean pH from 8.2 to 8.1 since pre-industrial times (Gattuso and Hansson, 2011). This phenomenon, commonly referred to as "ocean acidification", has raised considerable global concern about the current and future implications on marine life and seawater biogeochemistry. Model projections suggest that pH in the surface oceans could drop by another 0.13–0.42 units by the end of this century (Zheng and Cao, 2014).

The impact of ocean acidification on marine biota, both in terms of their biology and ecology, has been of particular interest (O'Brien et al., 2016). Most of the current research has focused on the effects of decreasing ocean pH on calcifying organisms (Ramajo et al., 2016; Hofmann et al., 2010), such as corals (Comeau et al., 2017), coccolithophores (Beaufort et al., 2011), foraminifera (Charrieau et al., 2018), marine invertebrates (Fabry et al., 2008; Garcia et al., 2008; Mevenkamp et al., 2018; Suckling et al., 2014) and coralline macroalgae (Koch et al., 2013; Hernandez et al., 2018) that form their shells and skeletons from calcium carbonate. Ocean acidification evidently affects biogenic calcification (Orr et al., 2005) and projected future CO_2 levels are expected to pose a serious threat to the survival of these organisms (Hofmann et al., 2010). A number of less dramatic effects have also been reported for non-calcifying species (Connell and Russell, 2010; Munday et al., 2009; Nunes et al., 2015), but our knowledge of how these organisms may respond to intensifying ocean acidification remains quite limited. This is also true for marine microorganisms, which dominate the

world's oceans in terms of abundance, diversity and metabolic activity (Pomeroy et al., 2007). By playing an integral role in global biogeochemical cycles (Arrigo, 2005), any major long-term perturbation in their physiology and biochemistry is likely to disrupt aquatic ecological balance (Crane and Grover, 2010).

What we know about the impact of ocean acidification on bacterial communities mainly stems from mesocosm (Allgaier et al., 2008; Riebesell et al., 2008; Endres et al., 2014; Oliver et al., 2014; Celussi et al., 2017) and microcosm (Krause et al., 2012) CO_2 perturbation experiments. Short study timescales and low replication are few of the limitations of these experimental approaches when attempting to provide realistic predictions of future impacts of chronic acidification (Barry et al., 2010). In this context, Hall-Spencer et al. (2008) proposed that submarine volcanic CO_2-venting areas can serve as natural laboratories for the *in situ* evaluation of marine biota responses to low pH. Such environments offer a unique opportunity to study the long-term consequences of ocean acidification and gain valuable information about the adaptability of microorganisms (Morrow et al., 2015).

The Kolumbo submarine volcano is a rather unexplored natural CO_2 venting site of Greece, situated 7 km northeast of Santorini island. Lying at the center of the Hellenic Volcanic Arc (Nomikou et al., 2012, 2013), it is one of the most active volcanoes in the eastern Mediterranean at present time (Nomikou et al., 2014; Ulvrova et al., 2016). Kolumbo is a completely enclosed crater with steep vertical inner slopes and a flat floor at 500 m depth (Fig. 1a) that is riddled with hydrothermal vents (Nomikou et al., 2012; Sigurdsson et al., 2006). Almost pure CO_2 (~99%) is continuously released from these vents, the dissolution of which causes a local pH reduction accompanied by an increase of water density (Carey et al., 2013; Christopoulou et al., 2016; Bakalis et al., 2017). The bowl-shaped morphology of Kolumbo impedes vertical mixing and leads to the accumulation of dense, CO_2-rich, acidic seawaters towards the deeper sections of the crater (Carey et al., 2013) .

This study aimed to assess the current status of natural acidification within Kolumbo crater and investigate whether the long-standing acidic conditions near the crater floor has any impact on the phenotypic traits of marine bacteria. To better evaluate the extent of this influence, the phenotypic tolerance of bacteria from the crater floor was compared to those isolated from overlying surface waters characterized by normal pH. For this purpose, a considerable number of bacteria were isolated from different seawater depths and a quantitative evaluation of acidity tolerance was conducted for strains belonging to the same genus. Considering the enriched polymetallic composition of hydrothermal chimneys and deposits at the crater floor (Kilias et al., 2013; Christakis et al., 2017), we also assessed the tolerance of bacterial isolates to several heavy metals. Since the hydrothermal vents have been recognized as "natural hot spots" of bacterial multiple resistances (Farias et al., 2015), we further examined whether tolerance to acidity and heavy metals is also accompanied by tolerance to other types of chemical stressors, such as antibiotics.

2. Materials and Methods

2.1. Seawater sampling and microbial strain isolation

Two sampling cruises were conducted with the research vessel R/V *Aegaeo* over the active hydrothermal vent area at the northern part of Kolumbo submarine volcano, in September 2013 and June 2014 (Fig. 1a). Niskin bottles were used to collect water samples from five (5, 45, 150, 250 and 495 m) and ten different depths (5, 20, 45, 60, 90, 120, 250, 430, 470 and 495 m) during the 2013 and 2014 campaign, respectively, and pH was measured on board using a precision pH meter (ProfiLine Multi 3320, WTW, Germany). Isolation of bacterial species was carried out on four surface (5, 20, 45 and 90 m) and two deep (430 and 495 m) seawater samples collected during the second campaign. For this purpose, four replicates (50 mL) from each depth were placed into sterile tubes and preserved at +4 °C until arrival at the

laboratory. Aliquots (100 µL) from each replicate were plated onto sterile Petri dishes containing marine agar 2216 (MA; BD Difco, MD, USA) and MA diluted 1:1 with artificial seawater (MA½). Inoculation of MA and MA½ plates was performed in triplicates. After incubation at 25 ^{o}C, plates were examined and single colonies were isolated and further purified by restreaking on fresh plates. Pure bacterial colonies were grown in marine broth 2216 and cells stocks were stored in 50% glycerol at -80 °C. A total of 83 strains were isolated.

2.2. Microbial strain identification

Extraction of genomic DNA from bacterial cultures was performed using the Wizard Genomic DNA Purification kit (Promega, WI, USA) following manufacturer's instructions. Purified DNA was quantified using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, USA) and 16S rRNA gene was amplified as described previously (Polymenakou et al., 2005). Sequencing was performed on an ABI 3730xl DNA analyzer using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, CA, USA) and the 27f primer (Lane, 1991). Partial 16S rRNA gene sequences were obtained and compared to those available in the GenBank database using the standard nucleotide-nucleotide BLAST algorithm to identify the closest relatives (Altschul et al., 1997). A total of 31 strains closely related to *Pseudomonas* were selected for further phylogenetic analysis and multi-stress tolerance testing. The partial 16S rRNA gene sequences of the isolates were deposited in GenBank database under accession numbers MG021215 to MG021245.

2.3. Phylogenetic analysis

Phylogenetic analysis was carried out using the partial 16S rRNA gene sequences of *Pseudomonas* isolates and of their closest relatives, yielding a single tree topology (n=56, average length = 400 bp). Sequences were aligned via ClustalW v.2.1 (Larkin et al., 2007) using gap opening penalty 7, gap extension penalty 2 for both pairwise as well as multiple

alignments, DNA weight matrix IUB and transition weight of 0.40. Bayesian statistics were then implemented in MrBayes v.3.2.6 (Ronquist and Huelsenbeck, 2003) to reconstruct the phylogeny. Bayesian analysis included four Monte Carlo Markov chains for 1,000,000 generations and a mixed model of nucleotide substitution with gamma-distributed rates among sites. Chains were sampled every 1000^{th} generation and the first 25% of generations were discarded as burn-in. The 50% majority-rule consensus was applied in order to generate the final tree topology with posterior probabilities of reconstructed clades. Tree topology was visualized via iTOL v.3.6.1 (Letunic and Bork, 2016) and edited using Inkscape v.0.91 (www.inkscape.org). The following statistics were obtained from Bayesian analysis: average standard deviation of split frequencies 0.0095, maximum standard deviation of split frequencies 0.036, average potential scale reduction factor 1.000, and maximum potential scale reduction factor 1.021.

2.4. Quantitative testing of bacterial susceptibility to various stressors

Bacterial tolerance was determined by evaluating bacterial growth in Lysogeny Broth (LB) cultures (pH=7.0) supplemented with increasing concentrations of the stress agents under investigation. All assays were performed in 384-well polystyrene microplates (Cat. #3702, Corning Inc, NY, USA) following the broth microdilution method described by Wiegand et al. (2008). For each isolate, a bacterial suspension was prepared in 2X LB and turbidity was adjusted to 0.5 McFarland (i.e. ~10^8 CFU mL^{-1}). The suspension was further diluted 1:100 with 2X LB to achieve a cell density of ~10^6 CFU mL^{-1}. For acid tolerance testing, 50-µL aliquots of bacterial suspension were loaded on a microplate, supplemented with varying volumes of HCl or NaOH solutions (5 to 60 mM) and diluted up to 100 µL with water to generate twelve microcultures of increasing pH (from 3.6 to 9.7; Supplementary Table S1). Additional microcultures were regularly prepared in a separate microplate and a precision pH-meter was used to verify that the desired pH values were achieved.

For screening of bacterial tolerance to heavy metals, 50-μL aliquots of As(III), Sb(III), Sr(II) and Hg(II) aqueous solutions (31, 30, 38 and 15 mM, respectively) were loaded on a microplate and serial two-fold dilutions in water were carried out in triplicate, followed by the addition of bacterial suspension (50 μL). Ten microcultures were prepared for each heavy metal to cover the following concentration ranges: As(III) 0.03−15.5 mM; Sb(III) 0.03−15 mM; Sr(II) 0.04−19 mM; Hg(II) 0.01−7.5 mM.

A similar approach was followed to evaluate the tolerance of bacterial isolates against six antibiotics. Stock solutions of ampicillin, erythromycin, ciprofloxacin, cefuroxime, tetracycline and chloramphenicol were prepared in appropriate solvent (EUCAST, 2003) and they were further diluted in water to obtain working solutions of desired concentration (280, 320, 4, 1200, 100 and 120 μg mL^{-1}, respectively). The latter were subjected to serial two-fold dilutions on a microplate and ten microcultures were prepared covering the following concentrations ranges: ampicillin 0.14−140 μg mL^{-1}; erythromycin 0.31−160 μg mL^{-1}; ciprofloxacin 0.004−2 μg mL^{-1}; cefuroxime 1.17−600 μg mL^{-1}; tetracycline 0.05−50 μg mL^{-1}; chloramphenicol 0.12−60 μg mL^{-1}. For five of the most tolerant and five of the most sensitive isolates, the experiments of antibiotic tolerance were repeated using 2X LB medium adjusted to pH=5.4 with HCl 0.5% v/v.

Mixing of the assay components (i.e. bacterial suspension, solutions of stress agents, water and 2X LB) in microplates and preparation of serial dilutions were performed by an automated liquid handling system (Biomek 2000; Beckman Coulter, CA, USA). Microplates were incubated at 37 °C for 22 hours and bacterial growth in each microculture was monitored by measuring optical density at 600 nm (OD_{600}) every 20 min using a microplate reader (Infinite F200 PRO, Tecan GmbH, Austria). The area under the growth curve (i.e. OD_{600} vs time) was integrated for each microdilution assay and the data were used for estimating the minimum concentration of each antimicrobial agent inhibiting 50% of

bacterial growth (MIC_{50}), as described elsewhere (Sorci et al., 2009). All tolerance experiments were performed in triplicate and the average MIC_{50} of each stressor was derived for each bacterial isolate. Growth controls (cell culture in LB without stressors) and sterility controls (LB medium) were also included in every microplate that was assayed. A total of 11,253 microcultures were performed in this study.

2.5. Statistical analysis

Statistical analysis was performed using Statistica v.8.0 (StatSoft Inc., OK, USA). Where MIC_{50} values fell below or above the range of concentrations tested, they were conservatively considered to be equal to the minimum or the maximum tested concentration, respectively. Data distribution was inspected by graphical assessment of normality (i.e. boxplots, Q-Q plots) and supplementary normality tests (i.e. Kolmogorov-Smirnov test, Shapiro-Wilk test). The average values were calculated to describe the central tendency of normally distributed data (i.e. seawater pH), while Student's t-test was used to evaluate the differences between two datasets. Similarly, the median value and Mann-Whitney U-test were applied for non-normally distributed data (i.e. MIC_{50}). Differences were considered significant when $p < 0.05$. The Spearman's rank correlation was used to assess the relationship between the MIC_{50} values of different stress factors, as the data tended to follow a non-normal distribution. Principal component analysis (PCA) was applied to assess whether bacterial isolates from different depths can be discriminated based on their multi-stress tolerance profiles. The MIC_{50} values of all studied stress factors were employed as variables and PCA was performed based on Spearman's correlation matrix.

3. Results

3.1. Seawater pH and temperature within Kolumbo submarine volcano

In both 2013 and 2014 sampling campaigns, the water column within Kolumbo crater

presented a considerable decrease of pH with increasing depth (Fig. 1b). Nevertheless, the topmost 150 m of the water column showed a remarkable pH stability across different depths and sampling periods, as the pH values were constrained between 8.20 and 8.24 providing an average of 8.21±0.01. During both campaigns, a pronounced decrease of pH was observed at a depth of 150 to 250 m, while subtle changes were evident from 250 m down to the crater floor. More specifically, the average pH in the water body below 250 m was 6.86±0.07 and 7.15±0.08 in 2013 and 2014, respectively. Although based on a limited number of measurements, the difference of seawater pH between the consecutive sampling events was deemed statistically significant (two-tailed t-test, p=0.01). Though, the pH difference between upper and lower water layers was of very high statistical significance (two-tailed t-test, p<0.001) during both sampling campaigns, The lowest measured pH value was 6.81 and it was recorded in 2013 at 495 m depth, just above the Kolumbo hydrothermal vent field. Seawater temperature showed limited variation with depth (17.7 to 19.2 °C; Fig. 1b) and it did not present a clear vertical gradient. The average temperature below and above 250-m depth was 18.1±0.3 °C and 18.7±0.5 °C, respectively, revealing no significant difference (two-tailed t-test, p=0.07).

3.2. Isolation and identification of culturable bacteria

A total of 83 bacterial colonies showing distinct morphological differences (i.e. color, shape, outline, texture and size) were isolated from Kolumbo water column, with 24 and 59 of them originating from the surface- (SSL; depth: 5 to 90 m) and deep-seawater layers (DSL; depth: 430 to 495 m), respectively. Sequencing of 16S rRNA gene revealed that 37% of the isolates retrieved throughout the water column were closely related to *Pseudomonas*. More specifically, a total of 31 isolates were attributed to this genus, with 10 and 21 of them stemming from SSL and DSL, respectively. The majority of *Pseudomonas* isolates (62%) were closely related to *Pseudomonas aeruginosa* (29%), *Pseudomonas stutzeri* (23%) and

Pseudomonas balearica (10%) (Supplementary Fig. S1). Considering their ubiquitous vertical distribution, the group of *Pseudomonas* strains was selected as a model to evaluate bacterial tolerance to different stress factors and enable a comparison between SSL and DSL isolates (Supplementary Table S2). Since the tolerance characteristics are frequently taxonomy-dependent (Dunivin et al., 2018; Barberán et al., 2017), focusing on a specific bacterial genus instead of all different isolates was also deemed necessary to minimize taxonomy-related biases in the present comparison.

3.3. Evaluation of bacterial susceptibility to acidic conditions

Acid tolerance of *Pseudomonas* isolates was investigated by examining their growth in liquid microcultures of decreasing pH. A considerable difference in acid tolerance was noticed between the strains retrieved from different water depths (Fig. 2a). In particular, the SSL strains exhibited a 50% growth inhibition at pH levels between 4.58 to 6.29, providing a median of 5.85, while the same inhibition effect was evident in DSL strains at substantially lower pH values (3.98 to 5.56; median: 4.60). Both SSL and DSL data indicated non-normal distribution, but non-parametric testing revealed a statistically significant difference in acid tolerance between the two groups (Mann-Whitney U-test, $p<0.001$). The 1.26 pH units difference between the medians implies that *Pseudomonas* strains from Kolumbo crater floor are able to cope with 18 times higher concentration of H^+ than the surface strains. It is worth mentioning that more than 50% of the DSL strains were able to grow at pH<5.0, while only 20% of the SSL isolates could tolerate such low pH conditions.

3.4. Evaluation of bacterial susceptibility to heavy metals

The isolates were also tested for their tolerance against four heavy metals (Fig. 2b–e) that were previously reported to be highly abundant in the polymetallic chimneys and hydrothermal deposits at Kolumbo seafloor (Kilias et al., 2013; Christakis et al., 2017). Regardless of water depth, the susceptibility of *Pseudomonas* strains against heavy metals

increased in the following order: Sr(II) < As(III) < Sb(III) < Hg(II). For all strains, the measured MIC_{50} (minimum concentration inhibiting cell growth by 50%) of Sr(II) (2.8−18.7 mM) and As(III) (0.04−3.0 mM) fell within the range of tested concentrations. On the contrary, the MIC_{50} of Sb(III) (<0.03−0.68 mM) and Hg(II) (<0.01−0.12 mM) were in several cases below the lowest concentration being tested. A comparison of heavy metal MIC_{50} values between SSL and DSL isolates did not reveal statistically significant differences for Sb(III) and Sr(II). Though, a higher tolerance to As(III) was observed for DSL (MIC_{50}: 0.16−3.0 mM, median: 0.40 mM) than SSL strains (MIC_{50}: 0.04−0.72 mM, median: 0.25 mM) and this difference was of statistical significance (Mann-Whitney U-test, p=0.04). The opposite, but also significant, trend (Mann-Whitney U-test, p=0.003) was revealed for the Hg(II) tolerance of DSL (MIC_{50}: <0.01−0.11 mM, median: 0.01 mM) and SSL strains (MIC_{50}: <0.01−0.12 mM, median: 0.04 mM).

3.5. Evaluation of bacterial susceptibility to antibiotics

Tolerance of isolates was tested against six commonly used antibiotics (Fig. 2f–k) representing six major classes of systemic antimicrobials (i.e. penicillins, amphenicols, tetracyclines, cephalosporins, quinolones; Coenen et al., 2009). Highly variable MIC_{50} values were observed for ciprofloxacin (<0.004−0.16 μg mL^{-1}), tetracycline (<0.05−2.06 μg mL^{-1}), ampicillin (<0.14 up to >140 μg mL^{-1}), chloramphenicol (0.20−11.8 μg mL^{-1}), erythromycin (<0.31−91 μg mL^{-1}) and cefuroxime (<1.17−566 μg mL^{-1}), with 25% of the results falling outside the range of tested concentrations. Based on median values, the susceptibility of *Pseudomonas* strains against antibiotics increased in the following order: cefuroxime ≈ erythromycin < chloramphenicol < ampicillin < tetracycline < ciprofloxacin. A comparison of MIC_{50} values with regard to water depth revealed striking differences for every antibiotic tested. In general, the DSL strains demonstrated a higher tolerance to all antimicrobials than the SSL strains. It is worth stressing that the median MIC_{50} values of erythromycin,

ciprofloxacin, chloramphenicol, tetracycline, cefuroxime and ampicillin were 7, 7.5, 12, 15, 39 and 457 times higher in DSL than SSL strains, respectively, with the majority of those differences being of very high statistical significance (Mann-Whitney U-Test, *p*-values of 0.02, <0.001, <0.001, <0.001, 0.02 and 0.003, respectively). Notably, the top 11 most tolerant phenotypes to each antibiotic were always bacterial isolates retrieved from deeper layers of Kolumbo crater (Supplementary Table S2).

3.6. Multi-stress tolerance profile of bacterial isolates

The MIC_{50} data from all nine stress factors were subjected to principal component analysis to elucidate any systematic differences in the multi-stress tolerance profiles of *Pseudomonas* isolates. The first two principal components (PC1 and PC2) explained approximately 52% and 19% of the total variance present in MIC_{50} dataset (Fig. 3). The PCA scores plot (Fig. 3a) revealed two distinct clusters with limited overlap, corresponding to the bacterial strains isolated from deep and surface seawaters. In essence, the majority of DSL isolates were scattered on the right side of the scores plot, while all SSL isolates were positioned on the opposite side (mainly in the lower left quadrant). Regarding the stress factors, all six antibiotics were clustered together on the right side of the loadings plot and showed high positive loadings in PC1 (Fig. 3b), while negative loadings were evident for pH and Hg(II). Moreover, the other three heavy metals formed a separate cluster with high positive loadings in PC2. Through a combined inspection of the scores and loadings plots, it was confirmed that the differentiation of DSL from SSL strains was mainly due to their higher tolerance to antibiotics and acidity, whereas their overall tolerance to heavy metals played a less prominent role.

We further attempted to determine the *Pseudomonas* isolates with the highest overall tolerance to acidity, heavy metals and antibiotics. For this purpose, the isolates were ranked with respect to the MIC_{50} of each stress factor and the species showing the highest overall

ranking were selected. Fig. 4 summarizes the top five isolates with the highest tolerance across the multiple stress factors, while the top five most susceptible strains are also presented for comparative purposes. All five of the most tolerant stains originated from deep seawater layers, while four out of the five most susceptible strains were retrieved from surface seawaters.

The interrelationships among the different bacterial tolerances were examined in more detail using the Spearman's rank correlation matrix (Supplementary Table S3). Strong positive correlations were observed between the MIC_{50} data of all six antibiotics (r: 0.49−0.90, *p*-value<0.005). On the contrary, only a few significant correlations were found among the four heavy metals. In particular, the MIC_{50} of Sb(III) exhibited weak positive correlations with those of As(III) and Sr(II) (r: 0.42−0.46, *p*-value: 0.010−0.017), while, Hg(II) showed a negative but also weak correlation with Sr(II) and Sb(III) (r: -0.39 to -0.43, *p*-value: 0.013−0.030). Bacterial tolerance to antibiotics and heavy metals indicated no correlation, with the exception of Hg(II), which showed moderate negative correlations with ampicillin, cefuroxime, tetracycline and chloramphenicol (r: -0.39 to -0.57, *p*-value: <0.001−0.030). More interestingly, strong negative correlations were found between the MIC_{50} data of pH and all six antibiotics (r: -0.53 to -0.77, *p*-value: <0.001−0.002), implying that isolates capable of tolerating high acidity levels were also tolerant to high levels of antibiotics. Acid tolerance indicated no relationship with heavy metals tolerance, excluding Hg(II) for which a weak positive correlation was observed (r: 0.55, *p*-value: 0.001).

The effect of low pH on MIC_{50} values was further examined for the five strains having either the highest or the lowest overall tolerance to antibiotics. All members of the latter subgroup were adversely impacted by reducing pH from 7.0 to 5.4 and cell growth was completely inhibited irrespective of antibiotic type and concentrations tested (Supplementary Table S4). Among the highly tolerant strains, M6.5 was the only one to show a concurrent decrease of

tolerance to all six antibiotics (i.e. between 12% and 90%) when the pH of culture medium was reduced to 5.4. On the contrary, the rest four strains showed a marked increase of tolerance to chloramphenicol, ciprofloxacin and erythromycin (on average 20%, 147% and 296%, respectively), while their tolerance to ampicillin remained steadily high. With regard to cefuroxime and tetracycline tolerance, the decrease of pH led to contradictory and inconclusive results.

4. Discussion

With a diameter of 3 km, Kolumbo is by far the largest submarine crater within the Santorini volcanic field (Nomikou et al., 2012). It last explosively erupted in 1650 A.D (Fouqué 1879; Cantner et al., 2014) and since then seismicity in the region is almost exclusively limited to the northeast trending Kolumbo volcanic line (Bohnhoff et al. 2006). Venting of high-temperature fluids (up to 220 ^{o}C) and vigorous gas emission plumes on the crater floor were firstly reported in 2006 (Sigurdsson et al., 2006), but the presence of large vent chimneys (up to 4 m in height) witnessed that hydrothermal discharges have been taking place for a very large time period. Later studies demonstrated that venting gases consisted of almost pure CO_2, the dissolution of which caused local increases in water density and the accumulation of acidic seawater close to crater's floor (Carey et al., 2013; Rizzo et al., 2016).

The results from our 2014 sampling campaign confirmed earlier observations (Carey et al., 2013) regarding the vertical increase of acidity from the upper to the lower layers of Kolumbo seawater column. In essence, the pH of surface layers (≤150 m depth) was typical of normal seawater conditions and approached the average global ocean pH (i.e. pH: 8.1) (Gattuso and Hansson, 2011), while it dropped by 1.1 pH units in deeper layers (≥250 m depth). The latter corresponded to H^+ concentrations that are ~13 times higher. Similar trends have been observed in previous years, though the vertical decrease of pH was even more

abrupt. In 2011, a decrease of 3.1 pH units was reported by Carey et al. (2013) between 100 and 500 m depth (i.e. from 8.1 to 5.0), while our team observed a vertical decrease of 1.4 pH units in a sampling campaign conducted in 2013 (i.e. from 8.2 to 6.8). Although the vertical profile of pH may be subject to significant temporal fluctuations due to the complex dynamics of hydrothermal systems and CO_2 degassing, it is clearly evident that deeper seawater layers in Kolumbo crater are consistently more acidic. On the other hand, temperature exhibited a quite uniform profile with water depth, implying that heat supply from seafloor hydrothermal process is efficiently dissipated and it is not intense enough to keep deeper water masses at notably higher temperatures.

Considering the well-known genetic plasticity of microbes, the present study aimed to ascertain whether persistent acidification across Kolumbo seawater column was able to drive natural selection and induce changes in bacterial tolerance against acidity and/or other types of chemical stressors. Although valuable insights about the environmental adaptability of microorganisms can be gained by genomic analysis, this approach is not a panacea as it can often be difficult to predict specific phenotypic traits from the presence/absence of particular genes alone (Barberán et al., 2017). With this in mind, the phenotypic evaluation of tolerance in cultivable bacteria was considered more appropriate and isolation of bacterial strains from different seawater depths was carried out for this purpose. Interestingly, a large number of isolates from both surface and deep layers were assigned to *Pseudomonas* genus. Considering the cosmopolitan character and the inherent adaptability to various and varying physicochemical conditions (Timmis, 2002; Koehorst et al., 2016), *Pseudomonas* was regarded as the ideal model microorganism for investigating the effects of acidification across Kolumbo crater.

A highly significant difference was observed in the acid tolerance of *Pseudomonas* strains isolated from surface and deep seawater layers (Fig. 2a). In comparison to surface, the

isolates from deep layers were able to grow in laboratory media with pH that was 1.3 units lower (based on median values). In particular, more than 50% of the deep-seawater isolates were capable of growing at pH lower than 5.0, while the respective percentage of surface isolates was only 21%. Apparently, these findings are directly related to the substantially higher acidity formerly experienced by the strains in deeper waters of Kolumbo submarine volcano, whereas they cannot be attributed to seawater temperature which showed only minimal changes with depth. To the best of our knowledge, this is the first study applying a cultivation-based phenotypic examination of bacterial acid tolerance in marine strains, obscuring a direct comparison with previous investigations. Up to now, mesocosm (Olive et al., 2014) and microcosm (Krause et al., 2012) experiments have been conducted to evaluate the effects of small pH changes on bacterial communities as a whole, providing contradicting conclusions regarding the actual impact of acidification. Although our findings cannot be generalized to the community level, they suggest that specific bacterial genera, such as *Pseudomonas*, are able to adapt even at large changes of pH.

Besides pH reduction, ocean acidification is expected to have an indirect effect on the solubility, adsorption and toxicity of heavy metals in aquatic systems (Millero et al., 2009). Overall, the solubility of heavy metals and their desorption from sediments and organic ligands are expected to increase, leading to elevated concentrations of dissolved metals into the water column (Millero et al., 2009; Ivanina and Sokolova, 2015). Considering the polymetallic composition of Kolumbo hydrothermal chimneys (Kilias et al., 2013; Christakis et al., 2017) and the increased acidity of surrounding seawaters, heavy metal concentrations and respective bacterial tolerances were expected to be greater near the crater floor. Our results indicated that, in comparison to the isolates from upper seawater layers, the deep-sea isolates had a higher tolerance to As(III) and Sb(III), although a statistically significant difference existed only for As(III). Surprisingly, the opposite trend was evident for Sr(II) and

Hg(II), but statistical significance was reached only for Hg(II). The basis of this finding is unclear, but we can speculate that hydrothermal fluids from Kolumbo crater floor are not as rich in Hg(II) as initially thought, while surface pollution sources of mercury (e.g. atmospheric deposition and/or soil runoff from nearby islands) (Gworek et al., 2016) may evoke higher Hg(II) levels in the upper water column, favoring the dominance of more Hg(II)-tolerant species. It is also intriguing that Kolumbo polymetallic chimneys have been previously reported to contain much higher levels of arsenic and antimony (average of 3.8 and 3.9 g Kg^{-1}, respectively) than strontium (1.8 g Kg^{-1}) and mercury (0.2 g Kg^{-1}) (Christakis et al., 2017). Moreover, it should be stressed that the tolerance levels of deep-sea strains to As(III) (0.16 − 3.01 mM) and Sb(III) (0.03 – 0.68 mM) were comparable to those previously reported for deep-sea isolates from other hydrothermal vents (1 mM As(III) and 0.5 mM Sb(III)) (Farias et al., 2015), while much higher values have been measured in bacterial isolates from terrestrial systems polluted with heavy metals (2 to 34 mM As(III) (Cai et al., 2009); 0.025 to 16 mM Sb(III) (Shi et al., 2013)).

There has been considerable speculation about possible genetic association between bacterial tolerance to heavy metals and multiple antibiotic resistance (Baker-Austin et al., 2006; Seiler and Berendonk, 2012; Pal et al., 2015). Here we report that bacterial isolates from Kolumbo crater floor exhibited higher tolerance to all tested antibiotics compared to isolates from surface seawaters. On average, the bacterial tolerance to ampicillin, cefuroxime, tetracycline, chloramphenicol, ciprofloxacin and erythromycin were 315, 38, 8, 7 and 5 times higher in deep-sea than surface isolates, respectively. The observed differences were statistically significant for all antibiotics ($p<0.05$) and particularly for ampicillin, tetracycline and chloramphenicol ($p<0.005$).

By applying principal component analysis on bacterial tolerance data, a distinct separation was observed between *Pseudomonas* isolates of surface and deep seawater layers. The

tolerance to high acidity and antibiotics were found to be the main differential parameters, while heavy metals played a less prominent role. More importantly, the isolates showing a higher tolerance to low pH values, they were also characterized by higher antibiotic tolerance. To our knowledge, this is the first time that a correlation between acidity and antibiotic tolerance is reported for marine bacterial isolates.

Furthermore, it was experimentally demonstrated that the majority of multi-tolerant strains originating exclusively from deep waters attained an even higher tolerance to antibiotics (i.e. chloramphenicol, ciprofloxacin and erythromycin) when the pH of culture medium was reduced from 7.0 to 5.4. On the contrary, the same decrease in pH caused complete inhibition of cell growth among the strains with the lowest multi-tolerance that were isolated from surface seawaters.

There is a building awareness about ocean acidification and how it might impact the marine environment and marine microbes (Joint et al., 2011; Weinbauer et al., 2011) in the years to come. The present study corroborates that *Pseudomonas* species isolated from a CO_2-rich marine environment are sufficiently flexible to cope with large changes of pH. It further demonstrates that the effects of acidification in submarine volcano are already visible in bacterial phenotypes, and indicates that submarine volcanoes can serve as an ideal natural laboratory for studying other aspects of ocean acidification. It also raises questions whether the ongoing phenomenon of acidification will have any considerable future implications on the antibiotic tolerance of bacterial communities or of specific bacterial strains. In this context, it is encouraging that the antibiotic tolerance of *Pseudomonas* strains from CO_2-rich (pH~6.8), deep waters of Kolumbo crater remains much lower compared to respective clinical strains (Molina et al., 2014). This finding suggests that even in the worst-case scenario for ocean acidification (i.e. drop of ocean surface pH to ~7.8 by 2100; Zheng and Cao, 2014), the foreseeable enhancement of antibiotic resistance in marine *Pseudomonas* is

unlikely to have any significant impact to human health, but it is impossible to make any speculation about the potential effects on marine life.

Based on our findings, a fairly rapid drop in the ocean's pH over the coming decades may not only favor the overall increase of Pseudomonas tolerance to antibiotics, but it may also cause the reduction or disappearance of their most acid-sensitive representatives. The natural populations of Pseudomonas in aquatic systems are thus likely to undergo a gradual community shift, but additional data are necessary to enable more accurate predictions. Further studies are required to assess if the development of co-tolerance to acidity and antibiotics is a caprice of *Pseudomonas* genus or a universal feature of marine bacteria, while additional research is needed to assess the long-run ecological consequences under the perspective of acidifying oceans.

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Author Contributions

M. Mandalakis and P.N.P. conceived the study. Sampling campaign with R/V *Aegaeo* was organized by P.N.P. (Chief Scientist on board). M. Mandalakis, C.A.C., P.N. and S.P.K. participated in the sampling campaign. Isolation of bacterial strains was undertaken by

C.A.C. and their genotypic characterization with 16S RNA gene analysis was performed by C.A.C. and P.N.P. The experiments for the determination of MICs were designed and supervised by M. Mandalakis, M.K and G.K. The implementation of experiments and the statistical analysis of MIC data were performed by A.G. The phylogenetic analysis of 16S rRNA gene sequences was performed by M. Medvecký and the bathymetric map of Kolumbo volcano was created by P.N. All authors contributed to interpretation of the results and the preparation of the manuscript.

Competing interests

The authors declare no competing interests.

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690 **Figure Legends**

691 **Figure 1.** Morphology of Kolumbo submarine volcano and vertical profile of pH within its
692 bowl-shaped depression. (a) High resolution swath bathymetric map of Kolumbo crater (the
693 yellow asterisk indicates the location of the sampling site). (b) Water column profile of pH
694 and temperature (°C) within the enclosed basin of Kolumbo crater, as recorded by the present
695 and previous studies (Carey *et al*., 2013).

696

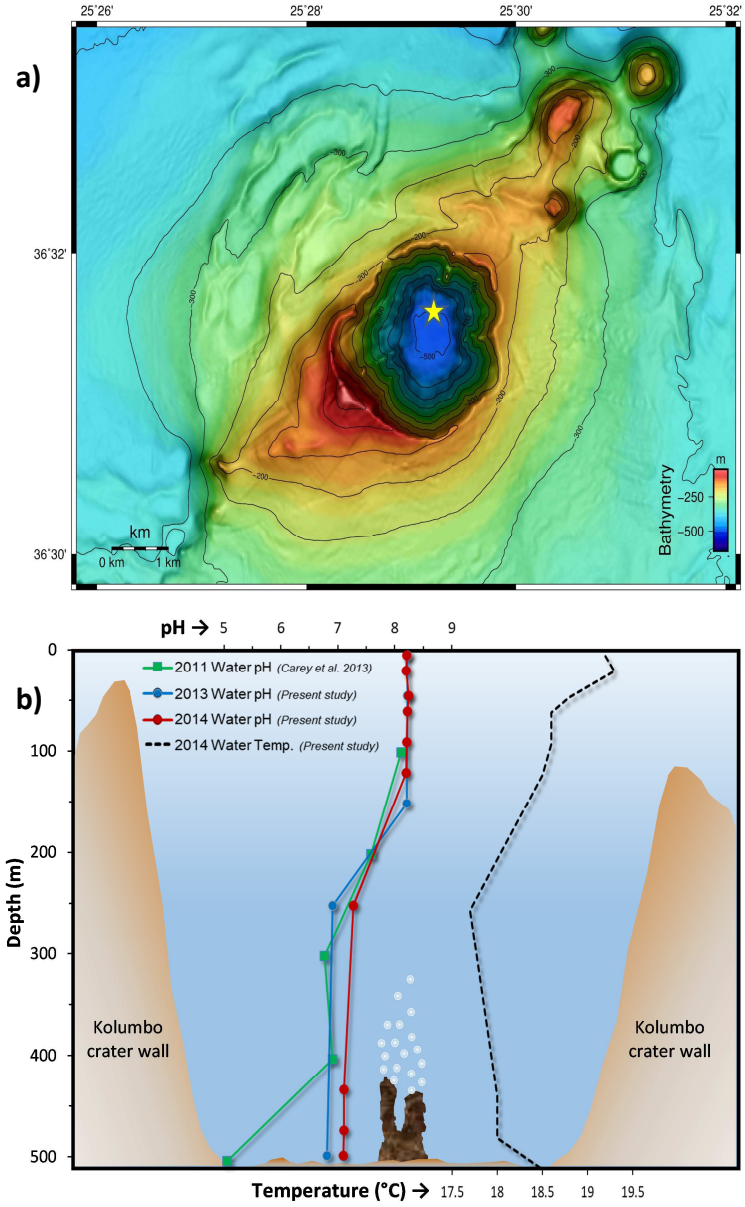
697 **Figure 2.** Tolerance of *Pseudomonas* strains isolated from surface- (SSL; N=10) and deep-
698 seawater layers (DSL; N=21) of Kolumbo submarine volcano against various stress factors
699 including (a) acidity (i.e. hydrogen ions), (b-e) heavy metals (Sr(II), As(III), Sb(III) and
700 Hg(II)) and six antibiotics (ampicillin, cefuroxime, erythromycin, chloramphenicol,
701 tetracycline and ciprofloxacin). Presented data correspond to the minimum concentration of
702 hydrogen ions (in pH units), heavy metals (in mM) and antibiotics (in μg mL^{-1}) required to
703 inhibit bacterial growth by 50% (i.e. MIC_{50}).

704

705 **Figure 3.** Principal component analysis a) score plot and b) loading plot for the MIC_{50} values
706 of antibiotics (ampicillin: Amp, erythromycin; Eryt, cefuroxime: Cef, ciprofloxacin: Cipr,
707 tetracycline: Tetr, chloramphenicol: Chlr), heavy metals (As(III), Sb(III), Sr(II), Hg(II)) and
708 acidity (i.e. pH) against the *Pseudomonas* strains isolated from surface and deep seawaters
709 across Kolumbo crater. Numbers in brackets represent the percentage of total variance
710 explained by each principal component.

711

712 **Figure 4.** Spider plots summarizing the MIC_{50} values of six antibiotics (in μg mL^{-1}), four
713 heavy metals (in mM) and acidity (in pH units) for the top-5 *Pseudomonas* isolates with a)
714 the highest and b) the lowest overall tolerance.


a)
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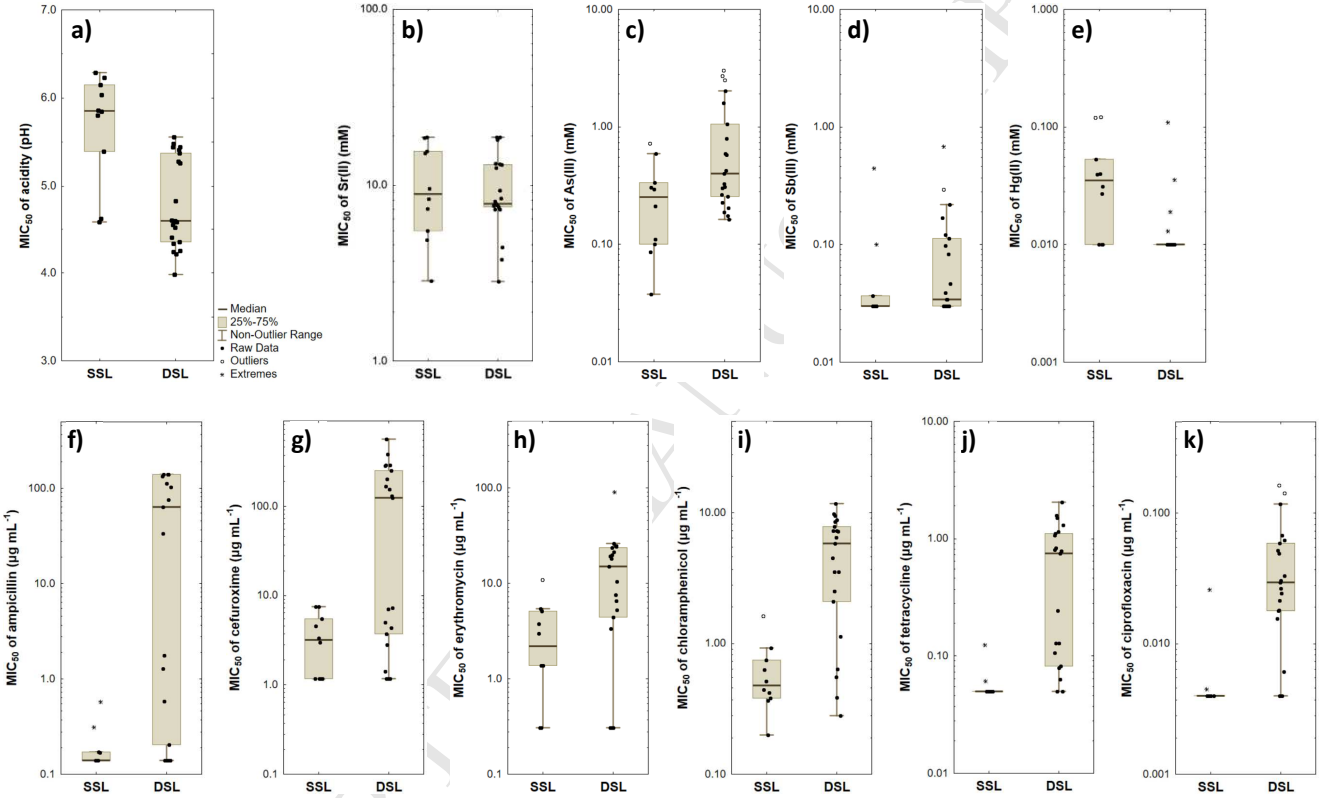
36°32'
36°30'
km
0 km
1 km
Bathymetry
m
-250
-500
b)
pH →
5
6
7
8
9
0
100
200
300
400
500
Depth (m)
2011 Water pH (Carey et al. 2013)
2013 Water pH (Present study)
2014 Water pH (Present study)
2014 Water Temp. (Present study)
Kolumbo crater wall
Kolumbo crater wall
Temperature (°C) →
17.5
18
18.5
19
19.5

Figure 1.


a)
b)
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h)
i)
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k)
MIC50 of acidity (pH)
MIC50 of Sr(II) (mM)
MIC50 of As(III) (mM)
MIC50 of Sb(III) (mM)
MIC50 of Hg(II) (mM)
MIC50 of ampicillin (µg mL-1)
MIC50 of cefuroxime (µg mL-1)
MIC50 of erythromycin (µg mL-1)
MIC50 of chloramphenicol (µg mL-1)
MIC50 of tetracycline (µg mL-1)
MIC50 of ciprofloxacin (µg mL-1)
SSL
DSL
Median
25%-75%
Non-Outlier Range
Raw Data
Outliers
Extremes

Figure 2.

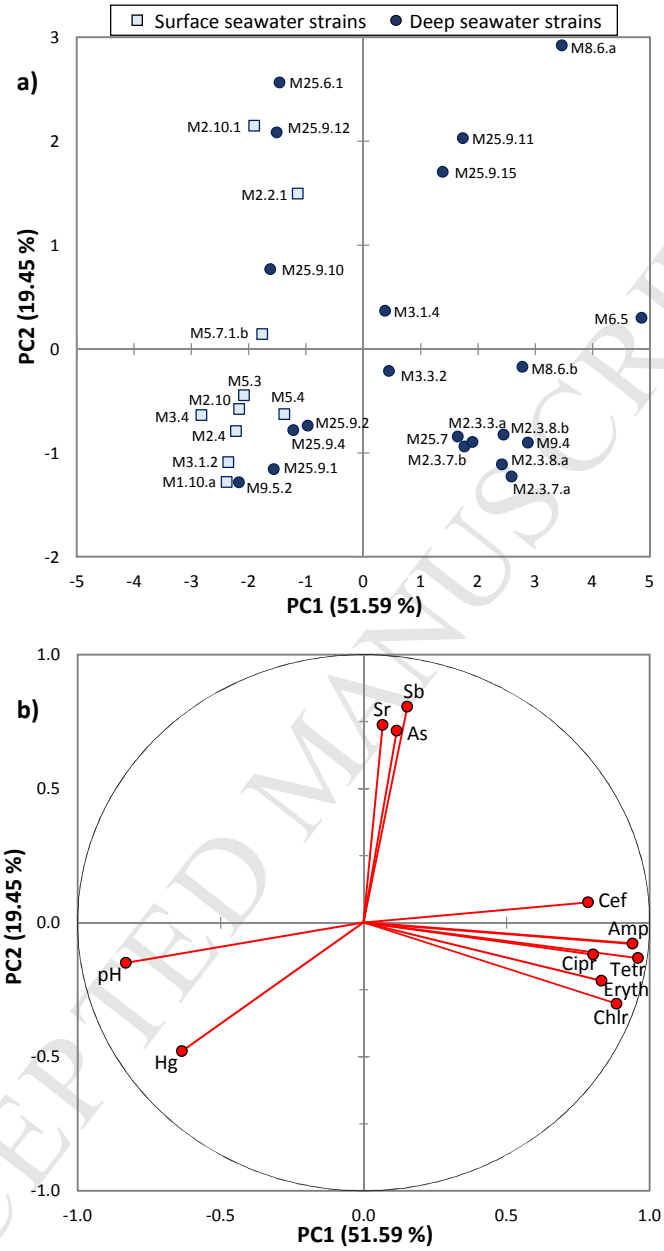

Figure 3.

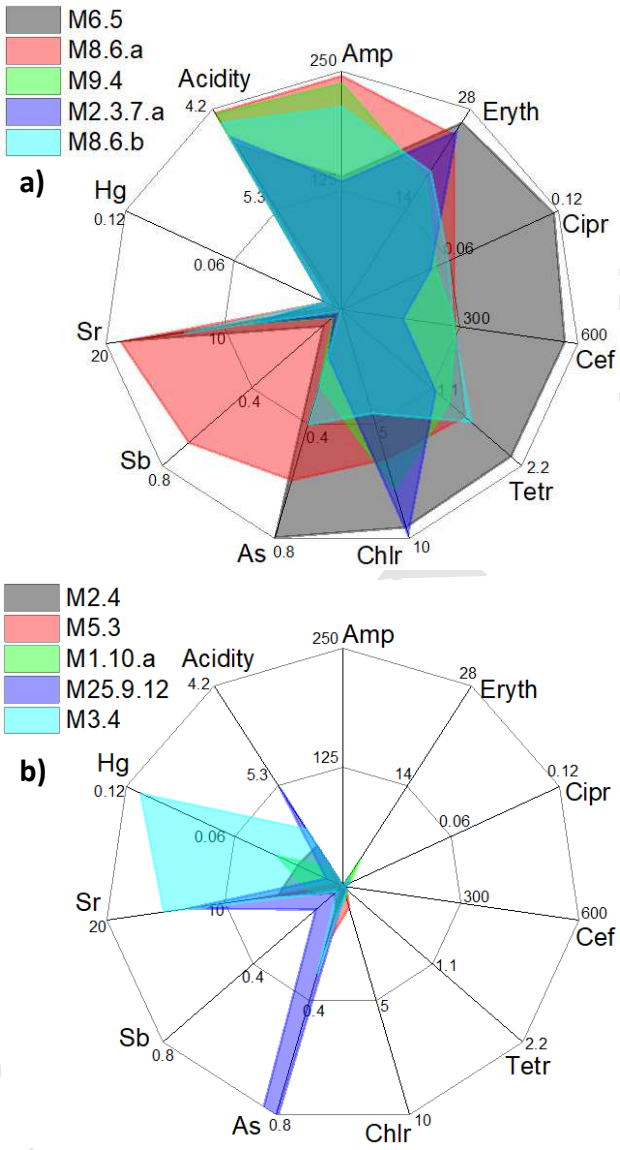
M6.5
M8.6.a
M9.4
M2.3.7.a
M8.6.b
a)
Amp
Eryth
Cipr
Cef
Tetr
Chlr
As
Sb
Sr
Hg
Acidity
250
28
0.12
600
2.2
10
0.8
20
0.12
4.2
125
14
0.06
300
1.1
5
0.4
10
5.3
M2.4
M5.3
M1.10.a
M25.9.12
M3.4
b)

Figure 4.

Highlights for MERE_2018_569

- The study investigates the effects of volcanic acidification to marine bacteria
- Deep waters of Kolumbo submarine volcano are CO_2-rich and more acidic
- Pseudomonas strains from Kolumbo seafloor show higher tolerance to acidity
- Strong correlation between acid and antibiotic tolerance of Pseudomonas species
- Ocean acidification may lead to marine bacteria with increased antibiotic tolerance