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Susceptibility of Linear and Nonlinear Otoacoustic Emission Components to Low-Dose Styrene Exposure

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Abbreviations:

OAE: Otoacoustic emission

TEOAE, DPOAE, SFOAE: Transient, Distortion Product, Stimulus-Frequency OAE

OHC: Outer-Hair Cell

TWA: Time-Weighted Average

WT: Wavelet Transform

QFC: Quadratic Frequency Coupling

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ABSTRACT

Objective: to investigate potential susceptibility of active cochlear mechanisms to low-level styrene exposure by comparing TEOAEs in workers and controls.

Design: Two advanced analysis techniques were applied to detect sub-clinical changes in linear and nonlinear cochlear mechanisms of OAE generation: the wavelet transform to decompose TEOAEs into time-frequency components and extract signal-to-noise ratio and latency of each component and the bispectrum to detect and extract nonlinear TEOAE contributions as quadratic frequency couplings (QFCs).

Study Sample: two cohorts of workers were examined: subjects exposed exclusively to styrene ($N = 9$) and subjects exposed to styrene and noise ($N = 6$). The control group was perfectly matched by age and sex to the exposed group.

Results: Exposed subjects showed significantly lowered *SNR* in TEOAE components at mid-to-high frequencies (above 1600 Hz) and a shift of QFC distribution towards lower frequencies than controls. No systematic differences were observed in latency.

Conclusion: Low-level styrene exposure may have induced a modification of cochlear functionality as concerns linear and nonlinear OAE generation mechanisms. The lack of change in latency seems to suggest that the OAE components where generation region and latency are tightly coupled may not have been affected by styrene and noise exposure levels considered here.

Introduction

Styrene is one of the most widely produced and used organic solvent in the world. It is typically employed as raw material to manufacture plastic products such as boats, utensils, furniture, and lightweight packaging. Inhalation of styrene in its volatilized form may have adverse health characteristics, such as carcinogenicity, genotoxicity, reproductive toxicity, and neurotoxicity (including ototoxicity) (IARC, 2002). Ototoxicity of styrene has been reported in experimental animals and humans (for a review of the main findings, see Fuente and McPherson, 2006; Lawton et al., 2006; Hoet & Lison, 2008; Triebig et al., 2009). In animals, inhalation of high levels of styrene (greater than 650-800 ppm) causes permanent elevation of hearing thresholds in the mid-frequency range and OHC loss (or damage) starting from the third row and following with the second and first rows, from the basal to the apical turn of the cochlea (see, e.g., the review of Hoet & Lison, 2008). In workers exposed to styrene alone or in combination with noise, significant association was found between styrene exposure and elevated hearing thresholds measured with behavioral and electrophysiological tests (Śliwińska-Kowalska et al., 2003; Fuente & McPherson, 2006; Lawton et al., 2006; Morata et al., 2011). Despite many reports, there is still no consensus on the lowest occupational exposure limit at which biologically significant increases in frequency or severity of adverse effects on the auditory system occur in the exposed population.

Only a few studies have specifically investigated the susceptibility of cochlear functionality to styrene. As reported above, animal studies suggested that styrene exposure is associated to OHC dysfunction. In humans, possible adverse effects on cochlear functionality has been investigated with otoacoustic emissions (OAEs), which are low-intensity sounds emitted by the healthy cochlea that indicate normal OHC function (Kemp, 1978). Thanks to their high test-retest reliability, OAEs are successfully utilized to monitor changes (also of subclinical

nature) in cochlear responsiveness to external agents such as low level noise exposure, electromagnetic fields, and ototoxic drugs (e.g., Tognola et al., 1999; Paglialonga et al., 2007; Janssen et al., 2000).

Findings on cochlear susceptibility to styrene exposure as inferred from OAE analysis are not consistent. In a number of studies, no direct and clear effect of styrene exposure was seen. For example, in a cross-sectional occupational study from (Hoffmann et al., 2006), which considered a group of 16 workers exposed to styrene at levels both below and above the exposure limit value, no significant differences were observed in TEOAEs amplitude and reproducibility measured in the control and in the exposed groups. Similarly, in another study of workers exposed to low (<10 ppm), medium (~10-20 ppm), and high (>20 ppm) styrene levels, no significant association of TEOAE signal to noise ratio (SNR) and amplitude was found with exposure data (Triebig et al., 2009).

In contrast, other studies found a significant susceptibility of TEOAEs and DPOAEs to styrene exposure. Johnson et al. (2006) compared DPOAE input/output functions measured at 4 kHz in workers exposed to styrene at levels below the recommended limit (20 ppm) and in a group of non-exposed workers. The two groups exhibit different DPOAE growth characteristics. Although there was no clear main effect of exposure on DPOAE level, a significant interaction between exposure and input level was seen: for input below 50 dB SPL, the group exposed to styrene had lower DPOAE levels than the controls, while for input higher than 50 dB SPL, the controls leveled off in contrast to the exposed group. Also, for input of 55-70 dB SPL, mean DPOAE levels were significantly correlated to hearing thresholds at 4 kHz in the control group, whereas no correlation was observed with hearing thresholds in the group exposed to styrene. Finally, in a cohort of workers (Sułkowski et al.,

2002) exposed to a mixture of solvents containing styrene at a concentration below the maximum accepted level set by the Polish national recommendations, DPOAE and TEOAE amplitudes were found to be inversely correlated with the dose of exposure: the higher the dose, the lower DPOAE and TEOAE amplitudes.

Many are the reasons that may explain why occupational studies on cochlear susceptibility to styrene exposure do not show consistent findings. One of the most crucial issue is the lack of homogeneity between compared groups in terms of size, age, exposure levels and additional confounding factors that may alter hearing functionality *per se*, such as presbycusis, combined exposure to noise, and/or additional ototoxic agents.

Also, in all the above mentioned studies the amplitude (or the SNR or the reproducibility) of OAEs was considered as the only relevant parameter for the quantitative measurement of the effects of styrene exposure, and OAE data were studied either for the whole emission frequency band or a number of narrower bands. Although these simple parameters are successfully used for reliable hearing assessment in clinical practice, they may not be sensitive enough to detect possible subtle dysfunctions or sub-clinical injuries. On the contrary, advanced and *ad hoc* analysis techniques which were based on the knowledge of generation mechanisms of cochlear emissions were successful in increasing OAE sensitivity to subtle cochlear dysfunctions, as demonstrated in (Tognola et al., 1999; Jedrzejczak et al., 2005; Paglialonga et al., 2011).

OAEs and, in particular, TEOAEs are the by-products of active and nonlinear amplification mechanisms in the cochlea. Some explanations are needed to avoid confusion between the nonlinearity of the basilar membrane (BM) response to acoustic stimuli and the possible

nonlinearity of the OAE generation mechanisms. Nonlinearity is one of the peculiar features of a healthy cochlea: the very early sign of cochlear damage is just that the system becomes more linear because of progressive loss of compression in the cochlear amplifiers (in OHC). As a result of the cochlear amplifier nonlinearity, the BM vibration level is a nonlinear compressive function of the stimulus level. Each TEOAE frequency component is the result of the interaction of two generation mechanisms: the nonlinear distortion and linear reflection (Shera et al., 1999). OAEs generated by a linear reflection mechanism are characterized by the same compressive dependence on the stimulus level typical of the BM displacement. OAE components generated by nonlinear distortion mechanism (the so-called intermodulation distortions) can be found at frequencies which are not present in the stimulus as they arise from interactions between frequencies present in the broadband stimulus (Withnell et al., 2008).

There has been a long discussion in the recent literature about the possible nonlinear distortion origin of the shorter latency component of TEOAEs and SFOAEs (Goodman et al., 2009). Although both numerical experiments (Moleti et al., 2013) and analysis of experimental data (Mertes & Goodman, 2013; Sisto et al., 2013b) seem to conclude that these OAE components come from linear reflection from more basal cochlear regions, the nonlinear distortion source cannot be ruled out, particularly at the rather high stimulus levels used in most TEOAE studies (including this one).

In this study, we applied two advanced analysis techniques to separate TEOAE into a set of frequency components, which may be associated to different characteristic frequency places. Although there could be various solutions that allow OAE of different frequencies to arise from locations on the BM not tonotopically associated with those frequencies, the link

between place of generation and frequency of OAE is backed by experimental data and a solid theoretical framework.

The first technique, based on the Wavelet Transform (WT), is a well-established advanced approach (Tognola et al., 1997) specifically designed for decomposing the TEOAE waveform into a set of frequency components, each representing cochlear contribution within a narrow region along the basilar membrane. This approach proved to be able to determine with a very high accuracy not only the frequency content but also the time course (e.g., the latency) of TEOAE components. It has been successfully employed in studies of cochlear susceptibility to potentially ototoxic factors (Tognola et al., 1999; Paglialonga et al., 2007; Jedrzejczak et al., 2005; Bhagat et al., 2013) and in cochlear subclinical dysfunctions (Paglialonga et al., 2011).

The second analysis technique employed in this study is based on the bispectrum and has been recently developed to detect and extract quadratic frequency coupling (QFC) components in TEOAEs (Tognola et al., 2013; Marchesi et al., 2013). This technique could be particularly of some help in studying OAE components arising from locations on the BM not tonotopically associated with their frequency. QFC components are the by-product of cochlear nonlinearities and consist of TEOAE components at a generic frequency f_3 which were originated by the interaction of two independent TEOAE components at frequencies f_1 and f_2 . To the best of our knowledge, no other approaches in the literature are able to directly extract QFCs from TEOAE signals. As illustrated in (Tognola et al., 2013; Marchesi et al., 2013), TEOAEs from newborns and adults are characterized by clear, strong and highly reproducible QFCs. Moreover, newborns and adult OAEs have different patterns of the distributions of QFC frequencies, with newborns showing QFCs in the range 3.5-4 kHz and adults mainly around 2 kHz. Analysis of QFCs extracted from TEOAEs recorded from young

adults in two different sessions and at increasing stimulus intensities (Tognola et al., 2013), revealed that the nonlinear interaction mechanism, which is responsible for the generation of QFC components, has a high test-retest repeatability. Moreover, it is susceptible to stimulus intensity: as the stimulus level increased, QFC components extracted from TEOAEs of the same ear shifted from high (2.5-3.0 kHz) to low (1.0-1.5 kHz) frequencies.

The present work extends a previous investigation (Sisto et al., 2013a) of cochlear susceptibility to low level styrene exposure, which covered two cohorts of workers of a fiberglass factory, one exposed to styrene alone and the other to styrene and noise. Workers in both cohorts exhibited TEOAE levels in 1/3-octave frequency bands and DPOAE levels in the range 1000-6400 Hz significantly different from the control group, which consisted of subjects with no exposure to noise and styrene. These results, although of valuable interest, must be interpreted with some caution, as age, sex distribution, and size of subjects in the control group were similar but not perfectly matched to those of the exposed group (the control group, for example, had a higher number of females and was slightly younger than the exposed group).

To further investigate the potential cochlear susceptibility to styrene with greater accuracy, the present work, while considering the same cohort of workers as in (Sisto et al., 2013a), extends the examination to two groups of controls (one for each cohort) perfectly matched by age and sex (and size) to exposed workers. As illustrated above, two analyses are performed: the first one, which employs the WT, is aimed to examine potential susceptibility to styrene of all OAE generation mechanisms. To this purpose, frequency components in the range 500-5000 Hz will be extracted and their time-frequency behavior will be analyzed both in term of their SNR and latency. The second analysis, which employs the bispectrum, is aimed to the

examination of only ‘nonlinear’ OAE generation mechanisms by investigating whether QFC components of TEOAEs of the control and the exposed groups exhibited the same patterns or are significantly affected by styrene exposure.

Materials and methods

Subjects

Exposed subjects were recruited from a fiberglass product manufacturing facility in Italy. Each subject was monitored during the working shift for both noise exposure (with a wearable sound level meter, Quest DLX-1, Quest Technologies, Wisconsin, USA) and styrene exposure (with a personal diffusive air sampler, Radiello, Sigma-Aldrich, Missouri, USA). The Time-Weighted Average (TWA) during the 8-h working shift was computed by averaging the data measured by the personal air sampler in two days. Workers recruited in this study are assigned to two working tasks: molding and artifact refining. Molding workers (N=9) were found to be exposed to levels of airborne styrene approximately on the order of the Threshold Limit Value (TLV) of 85.2 mg/m³ set by the American Conference of Governmental Industrial Hygienists (ACGIH, 2014) and low levels of noise, below the action level of 85 dBA set by the Occupational Safety & Health Administration. Artifact refining workers (N=6) were found to be exposed to lower levels of airborne styrene than the molding workers (<< 10 mg/m³) and high levels of noise, above 85 dBA. Molding and refining workers will be analyzed separately and will be named as the “Styrene” group (S_E) and the “Noise & Styrene” group (NS_E), respectively.

Two separate groups of subjects with no exposure to styrene and noise and perfectly matched by age and sex to exposed subjects were recruited as controls. Control group S_C consisted of nine adults matched to subjects in group S_E, whereas control group NS_C consisted of six

adults matched to subjects in group NS_E. Control and exposed groups were assigned to two analysis cohorts: cohort 1, consisting of groups S_E and S_C and cohort 2, consisting of groups NS_E and NS_C. Details on individual characteristics of all groups are reported in Table I.

For all subjects, audiometric thresholds (PTA) were evaluated for both ears at 0.5, 1, 2, 3, 4, 6, and 8 kHz, starting from the level of 20 dB HL and compared to thresholds expected in a population of otologically normal people matched for age and sex, as described in the ISO7029 standard (ISO, 2000). As a general remark, in all the examined ears, no audiometric thresholds exceeded 30 dB HL at any frequency. Table II shows for each subject which frequency had audiometric thresholds higher than those reported in the reference ISO7029 standard. In group S_e (N=18 ears), we found thresholds higher than those expected in a population of otologically normal people matched by age and sex, as described in the ISO7029 standard (ISO, 2000), at 500 Hz (in eight ears), 4 kHz (in four ears), and 6 kHz (in two ears). In group NS_e (N=12 ears), we found thresholds higher than those reported in the reference ISO7029 standard at 500 Hz (in eight ears) and at 1 kHz (in two ears). In contrast, in both control groups, no subjects with hearing thresholds higher than those reported in the reference ISO7029 standard at any audiometric frequency were found.

TEOAE recording

TEOAEs were recorded by means of a standard adult probe using an Otodynamics ILO292 device. TEOAEs were elicited by click stimuli of 80(±3) dB SPL peak equivalent using the “nonlinear” acquisition mode (consisting of a train of three clicks followed by a click of greater amplitude and inverted polarity). Responses were digitized at a sampling rate of 25 kHz, stored in two 20.5 ms (512 samplings) buffers A and B until 260 repetitions of the click stimuli were acquired in each buffer, and then averaged. Noise rejection level was set equal to

6 mPa; for all recordings, stimulus stability was $\geq 95\%$. Measurements were carried out in a silent room. The overall level of background noise was comparable in all the TEOAE recordings. As an example, Fig. 1(a) shows superimposed the two replicate TEOAE recordings stored in buffers A and B of a control subject.

TEOAE analysis

LINEAR ANALYSIS

To analyze TEOAEs we applied the WT method to decompose TEOAEs in a set of narrowband time-frequency components in the range 500-5000 Hz (for more details, see Tognola et al., 1997). The WT at the generic time t and frequency f of signal $x(t)$ is defined as (Mallat, 1989):

$$WT(\tau, f) = \int_t x(t) \sqrt{\frac{f}{f_0}} \psi^* \left(\frac{f}{f_0} (t - \tau) \right) dt \quad (1)$$

where $*$ denotes complex conjugation. Basically, the WT decomposes a signal $x(t)$ into elementary components by means of a bank of band-pass filters - $\sqrt{f/f_0} \cdot \psi(f/f_0(t))$ - that are iteratively derived from a unique prototype function $\psi(t)$, called ‘mother wavelet’. The mother wavelet is a function with finite energy, centered around time $t = 0$ and its Fourier Transform is a bandpass function centered around frequency f_0 . The bandwidth of the band pass filters derived from the mother wavelet is proportional to the central frequency f of the filter, whereas the duration of their impulse response in the time domain is inversely proportional to the central frequency of the filter (for a more detailed mathematical treatment, see Tognola et al. (1997). Roughly speaking, this type of analysis provides a decomposition of a signal into sub-bands with a bandwidth linearly increasing with frequency.

By using Eq. (1), each TEOAE waveform was decomposed into 100-Hz wide (exact bandwidth 97.66 Hz) WT frequency components $WT(\tau, f_k)$, with f_k ($k = 1, 2, \dots, K$) in the approximate 500-5000 Hz range (exact frequency range 488-5078 Hz), for a total of $K = 48$ components. As an example, Fig. 1(b) shows the WT decomposition of the TEOAE recording of the control subject displayed in Fig. 1(a). Each row of Fig. 2(b) show superimposed the frequency components $WT_A(\tau, f_k)$ and $WT_B(\tau, f_k)$ extracted at frequency f_k from the two TEOAEs replicate recordings A and B. For sake of clarity, only ten out of the 48 calculated WT components were displayed.

The main parameters evaluated for each TEOAE component were the signal-to-noise ratio (SNR_k) and the latency (LAT_k) of the 48 frequency components. SNR is one of the most widespread TEOAE parameters used both in basic research and in clinical practice in differentiating normal hearing from hearing impaired subjects (see, e.g., Gorga et al., 1993; Hussain et al., 1998; Harrison and Norton, 1999; Prieve et al., 1993; Tognola et al. 1999) and in analyzing the effects of exposure to organic solvents (Triebig et al., 2009). As described in previous studies (Gorga et al., 1993; Hussain et al., 1998; Harrison and Norton, 1999), as SNR is a normalized parameter that takes into account the level of noise, it allows to reduce the confounding effect of inter-subject variability in noisy recordings and thus allows to obtain more powerful statistical tests. Also, the use of SNR could ease the comparison of results obtained in this study with findings from previous studies about styrene and noise exposure (see e.g. Triebig et al., 2009). In particular, SNR_k is defined as the difference between the dB SPL value of the energy of the signal and noise of each WT component at frequency f_k . For each of the 48 WT frequency components, the signal has been defined as $0.5 * (WT_A(\tau, f_k) + WT_B(\tau, f_k))$ and the noise is defined as $(WT_A(\tau, f_k) - WT_B(\tau, f_k)) / \sqrt{2}$.

The latency LAT_k of each of the 48 frequency components has been defined as the time

interval between the stimulus onset and the time sample where the envelope of the signal $0.5 * (WT_A(\tau, f_k) + WT_B(\tau, f_k))$ reached its maximum value. For example, numbers displayed on the left side of Fig. 1(b) are the SNR_k and LAT_k values of each of the ten displayed WT components.

NONLINEAR ANALYSIS

The second TEOAE analysis, based on the bispectrum, is aimed at investigating nonlinear TEOAE generation mechanisms. The bispectrum (Nikias & Raghuveer, 1987) is a method able to identify nonlinear components in a real, stationary, ergodic and discrete-time signal $x(k)$ ($k = 0, 1, 2, \dots, N$). It has been calculated using the so-called third-order scaled polyperiodogram:

$$\hat{M}_3^x(f_1, f_2) \triangleq \frac{1}{N^3} X(f_1)X(f_2)X^*(f_1 + f_2). \quad (4)$$

where $*$ is the complex conjugate and $X(f)$ is the Fourier transform of $x(k)$. Roughly speaking, the bispectrum is the triple product of the Fourier transform evaluated at two frequencies f_1 and f_2 and at their sum. The set of $\hat{M}_3^x(f_1, f_2)$ values obtained for pair frequencies f_1 and f_2 tell us whether the component of signal $x(k)$ at the generic frequency $f_3(=f_1+f_2)$ has been generated by the interaction of the two independent components at f_1 and f_2 and gives us an indication on how strong is this interaction. By way of example, Fig. 1(c) and Fig. 1(d) show, respectively, the frequency spectrum obtained by classic Fourier analysis and the bispectrum (as estimated by the third-order scaled polyperiodogram $\hat{M}_3^x(f_1, f_2)$) of the TEOAE recording of Fig. 1(a). The Fourier spectrum is reported here as an example of classical spectral analysis compared to the bispectral analysis. As displayed in Fig. 1(c), the main spectral components of the TEOAE of Fig. 1(a) are in three frequency regions, i.e. around 1 kHz, 2.3-2.5 kHz, and 2.7-2.8 kHz. The lower part of Fig. 1(d) shows the contour plot of the values of $\hat{M}_3^x(f_1, f_2)$ in the (f_1, f_2) plane. $\hat{M}_3^x(f_1, f_2)$ is distributed mainly in the

region $[f_1 \leq 3, f_2 \leq 1.5]$ kHz and exhibits four main peaks at (1.3, 1.0) kHz, (1.1, 1.0) kHz, (1.4, 1.3) kHz, and (1.7, 1.0) kHz, thus suggesting the presence of QFC components at $f_3 = 2.3, 2.2,$ and 2.7 kHz.

As demonstrated in our previous studies (Tognola et al., 2013; Marchesi et al., 2013), a robust, stable and reliable parameter to investigate cochlear nonlinear generation mechanisms in TEOAEs is the amplitude $AQFC_{f_3}$ of the QFC component at frequency f_3 , which is defined as the sum of $\widehat{M}_3^x(f_1, f_2)$ values for all the (f_1, f_2) pairs where $f_1 + f_2 = f_3$.

Data analysis

For statistical analysis purposes, the 48 SNR_k and LAT_k values were averaged into ten 1/3-octave frequency bands with center frequencies f_j ($j = 1, \dots, 10$) in the range 500-4000 Hz. To prevent unreliable results, only LAT values calculated from 1/3-octave bands with $SNR \geq 3$ dB were considered. This threshold of 3 dB was set to avoid the calculation and the subsequent analysis of LAT values on bands that, as revealed by their poor SNR , did not show a real cochlear contribution. To preserve the perfect matching by age and sex between control and exposed groups, we considered only those LAT values that, for a given 1/3-octave frequency band, passed the selection rule both in the control and in the exposed group.

Similarly to SNR_k and LAT_k , $AQFC_{f_3}$ was integrated in the same ten 1/3-octave wide bands and then normalized to the total energy of the bispectrum $\widehat{M}_3^x(f_1, f_2)$ as follows:

$$AQFC(f_j) = \frac{1}{\sum_{f_1} \sum_{f_2} \widehat{M}_3^x(f_1, f_2)} \sum_{f_3 = L_{fj}}^{H_{fj}} AQFC_{f_3} \quad (5)$$

where L_{fj} and H_{fj} are the low and high frequency limits of the j^{th} 1/3-octave band. Fig. 1(d)

shows $AQFC$ values (black circles) calculated by Eq. (5), i.e. by integrating $\widehat{M}_3^x(f_1, f_2)$ in the 1/3-octave frequency bands displayed in Fig. 1(d) as dashed counter-diagonals.

Finally, to investigate the potential effect of age on SNR , LAT and $AQFC$, subjects of the first cohort were divided in three subgroups (<40 yrs, 40-44 yrs, ≥ 45 yrs), while subjects of the second cohort were divided in two subgroups (<40 yrs, ≥ 40 yrs).

Statistical analysis of left and right ear asymmetry: a two sample t-test was applied for each frequency band to check any possible asymmetry of TEOAE parameters extracted from recordings of left and right ears. The test revealed that the parameters came from independent random samples from normal distributions with equal means. As such no asymmetry was found, so right and left ears were analyzed together.

The Kolmogorov-Smirnov test for normality was performed on SNR , LAT and $AQFC$, to verify the assumption of normality required to perform analysis of variance. A 3-way analysis of variance (ANOVA) was applied to each cohort to test the effect of exposure, frequency band and age. Where significant differences were found, *post-hoc* analysis was performed using Tukey's test for multiple comparisons. Values of $p < 0.05$ were considered as statistically significant.

Results

For sake of clarity, results will be described separately for each of the three features considered in this study.

Feature #1: SNR

Table III shows the results of ANOVA tests performed on the three features – *SNR*, *LAT*, and *AQFC* – for the two cohorts. For all three features and both cohorts, no significant interaction among the three factors (group x frequency x age) was observed. For both cohorts, ANOVA tests revealed that the *SNR* varied significantly between groups (control vs. exposed) ($F_{1,359}=10.62, p \leq 0.01$ for cohort 1; $F_{1,239}=7.59, p \leq 0.01$ for cohort 2) and among frequency bands ($F_{9,359}=14.81, p \leq 0.001$ for cohort 1; $F_{9,239}=11.80, p \leq 0.01$ for cohort 2) but no significant effect of age was observed. Also, for subjects in cohort 1, a significant interaction between group and frequency ($F_{9,359}=2.16, p \leq 0.05$) was found. Fig. 2 displays the average *SNR* values in 1/3-octave bands of all four groups (S_E / S_C / NS_E / NS_C) of the two cohorts. As anticipated by the ANOVA tests (see Table III), it can be seen that for both cohorts *SNR* varies among frequency bands and between groups; in particular, for what concerns the group effect, subjects in the exposed groups S_E (styrene alone) and NS_E (styrene&noise) exhibited *SNR* values lower than in control groups S_C and NS_C . As to cohort 1, Fig. 2 also revealed that the distribution of *SNR* values in the frequency bands in control subjects (S_C) and in subjects exposed to styrene (S_E) followed different patterns: in group S_E , *SNR* increased at 1000 and 1250 Hz and steeply decreased in bands centered at the lowest (≤ 800 Hz) and highest (≥ 2500 Hz) frequencies. In contrast, in group S_C , *SNR* was high in the mid-to-high frequency bands from 1000 to 4000 Hz and steeply decreased in the lowest frequency bands, below 800 Hz. Finally, Fig. 2 shows that components at frequencies ≥ 2 kHz in group S_C were characterized by higher *SNR* values than in group S_E . Table IV displays in details for all four groups ($S/C_S/NS/C_{NS}$) of both cohorts the results of the *post-hoc* tests on the significant (group x frequency) interactions previously shown in Table III. As to *SNR* (first two columns of Table IV), *post-hoc* tests confirmed that the different distributions of *SNR* between groups S_E and S_C observed in previous Fig. 2 were significant. As to group S_E , *SNR* of the first two 1/3-octave bands centered at $f_1=500$ Hz and $f_2=625$ Hz was significantly different from the *SNR* of the

mid-frequency bands from $f_3=800$ Hz to $f_7=2000$ Hz; SNR from $f_4=1000$ Hz to $f_5=1250$ Hz was significantly different from that measured in the highest frequency bands from $f_8=2500$ Hz to $f_{10}=4000$ Hz. As to group S_C , SNR at frequencies $f_1=500$ Hz and $f_2=625$ Hz was significantly different from that measured at frequencies from $f_3=800$ Hz to $f_{10}=4000$ Hz. *Post-hoc* tests on SNR distributions for cohort 2 were not performed, as for this cohort there was no significant (group x frequency) interaction.

Also, Table III shows that there was a significant (group x age) interaction in SNR values of both cohorts. As detailed in Table V, the youngest subjects (aged less than 40 yrs) of the exposed group of cohort 1 exhibited significantly ($p \leq 0.05$) lower SNR values than their peers in the control group, whereas no significant differences were found between SNR values of the exposed and control group for subjects aged more than 40 yrs. For what concerns cohort 2, the oldest subjects in the exposed group exhibited significantly lower SNR values than their peers in the control group; no significant differences were found between the youngest (≤ 40 yrs) subjects of the exposed and control groups. It is worthwhile to remind that control groups of both cohorts consist of subjects that were perfectly matched by age and sex to their peers in the corresponding exposed groups.

Feature #2: LAT

As reported in Table III, ANOVA tests revealed that LAT varied significantly between groups (control vs. exposed) of only cohort 1 ($F_{1,211} = 4.45, p \leq 0.05$) and among frequency bands for both cohorts ($F_{7,211} = 52.82, p \leq 0.001$ for cohort 1; $F_{4,91} = 21.12, p \leq 0.001$ for cohort 2); no significant main effect of age was observed in both cohorts. For both cohorts, ANOVA revealed no significant (group x frequency) interaction (see Table III).

Fig. 3 shows LAT values averaged into 1/3-octave bands for groups S_E and S_C of cohort 1 and for groups NS_E and NS_C of cohort 2. One should note that the definition of latency used in the present study selects a single value in each frequency band. This value can be seen as the result of the combination of two different latency OAE components (Goodman et al., 2009; Sisto et al., 2013b), characterized by different growth rates, with the shorter latency components growing faster with increasing stimulus level than the longer latency components. As the stimulus level increases, the shorter latency components become dominant, which is explained by the fact that the basilar membrane excitation profile becomes less strongly peaked as the stimulus level increases. This phenomenology may be appreciated by measuring the TEOAE latency as a function of the stimulus level (Sisto et al., 2007). The same characteristic behavior could be expected as a result of cochlear impairment, because damage of the cochlear amplifier also yields less peaked basilar membrane excitation patterns, for a given stimulus level. If this is the case, the latency of impaired ears should be typically shorter, because the “center of gravity” of the OAE generation region would shift towards the base, favoring the shorter latencies components.

In the exposed and control subjects of both cohorts, LAT values followed the characteristic trend of higher frequencies having shorter latencies and lower frequencies having longer latencies. In normal hearing subjects, this trend between latency and frequency is well described by a power law model, as shown by Tognola et al. (1997):

$$LAT = a \cdot f^b$$

where LAT and f are the latency in (ms) and the frequency (in kHz) of the TEOAE component, and a and b are constant parameters. Normative data provided in (Tognola et al., 1997) showed that a depends on the intensity of the click stimuli, whereas b is almost stable

with stimulus intensity and, in adults with well-functioning OHCs, typically ranges from 0.44 to 0.40.

Feature #3: AQFC

Results of ANOVA of *AQFC* values of both cohorts are displayed in Table III. For both cohorts, *AQFC* varied significantly among frequency bands ($F_{9,359} = 63.25, p \leq 0.001$ for cohort 1; $F_{9,239} = 62.00, p \leq 0.001$ for cohort 2); in contrast, no significant main effect of group (exposed vs. control) and age was found, meaning that, on average, *AQFC* values measured in exposed and control groups and among age groups were not significantly different. However, when data were analyzed locally, frequency by frequency, a significant (group x frequency) interaction was found in both cohorts. As displayed in Fig. 4 and further confirmed by *post-hoc* comparisons reported in Table IV, significant differences were found in the patterns of *AQFC* distribution in frequency bands in exposed and control subjects. In exposed subjects (group S_E of cohort 1 and NS_E of cohort 2), frequency bands in the range 2000-4000 Hz were characterized by the highest contribution of QFC components, whereas frequencies in the range 500-1600 Hz had a steep decrease in *AQFC*. In control subjects (group S_C of cohort 1 and NS_C of cohort 2), high *AQFC* values were observed in a range slightly shifted towards higher frequencies than in exposed subjects, i.e., between 2500 and 4000 Hz; frequency bands below 2000 Hz exhibited a marginal contribution to QFC components, lower than that observed in exposed subjects. Results from *post-hoc* comparisons within groups displayed in Table IV confirmed that differences in *AQFC* distributions in exposed and control subjects of both cohorts were significant: in the exposed subjects (see column 3 and 5), bands at frequencies from $f_1 = 500$ Hz to $f_5 = 1250$ Hz have significantly ($p \leq 0.05$) lower *AQFC* values than bands in the range from $f_6 = 1600$ Hz to $f_{10} =$

2500 Hz; in the control subjects (see column 4 and 6), bands from $f_1 = 500$ Hz to $f_6 = 1600$ had significantly lower *AQFC* values than bands in the range $f_7 = 2000$ Hz to $f_{10} = 2500$ Hz.

Discussion and conclusions

The present study investigates potential cochlear susceptibility to styrene alone and in combination with noise by comparing TEOAEs in exposed workers and in control subjects with no exposure. To avoid possible confounding effects when comparing the data, the control group was perfectly matched by age and sex (and, thus, size) with the exposed group.

WT method and bispectral analysis were applied to TEOAEs to extract a set of features useful to detect sub-clinical changes in linear and nonlinear interaction cochlear mechanisms responsible of OAE generation.

Although workers were exposed to styrene levels below the TLV, which represents the “condition under which it is believed that nearly all workers may be repeatedly exposed, day after day, over a working lifetime, without adverse health effects” (ACGIH, 2014), the proposed analysis procedure revealed differences at a sub-clinical but statistically significant level between TEOAEs of exposed and control subjects. On average, on the overall range 500-4000 Hz, TEOAE components of the exposed groups had significant lower SNRs than the control group. On a local, frequency-by-frequency basis, differences between exposed and control subjects were mainly observed for TEOAE components at mid-to-high frequencies, above 1600 Hz (see Fig. 2). This finding was observed both in workers exposed to styrene alone (cohort 1) and in those exposed to styrene and noise (cohort 2). The *SNR* is a measure of the strength of a TEOAE in its frequency components and is known to significantly decrease in hearing impaired ears (see, e.g., Prieve et al., 1993; Tognola et al. 1999; Lucertini et al.,

2002; Moleti et al., 2005). Interestingly, the observed differences of *SNR* at mid-to-high frequencies of TEOAEs of exposed and control subjects seems to parallel what has been observed in animal exposed to styrene (Hoet & Lison, 2008), that is OHC loss (or damage) started from the basal to the apical turn of the cochlea, i.e., from high to low frequencies. As to former human studies employing TEOAE analysis for the assessment of cochlear susceptibility to styrene, current *SNR* results confirm what was observed in the previous work of (Sisto et al., 2013a). In (Sisto et al., 2013a) authors found that exposed subjects had significant lower TEOAE levels than control subjects, especially at the mid-to-high frequency bands. It is worthwhile to remind here that our study and that of (Sisto et al., 2013a) investigated the same groups of workers, but, differently from (Sisto et al., 2013a), our study recruited a group of control subjects perfectly matched by age and sex to group of workers and employed a different analysis procedure to extract TEOAE frequency bands. Similar to our results, Sułkowski et al. (2002) found significantly lower level in the broadband TEOAE signal of exposed subjects than in control subjects. In contrast, our study seems to provide opposite results to those published by Hoffmann et al. (2006), who did not observe any significant difference in the amplitude of the broadband TEOAE signals of workers exposed to styrene and control subjects. Direct comparison with our results is, however, not straightforward as both Hoffmann et al. (2006) and Sulkowski et al. (2002) draw their conclusions only on the basis of what they observed in the global broadband TEOAE signal and neither of them performed any TEOAE analysis in narrowband components. Moreover, Hoffmann et al. (2006) and Sulkowski et al. (2002) involved workers exposed to styrene levels different from our subjects. Finally, the study by Triebig et al. (2009) did not find any significant association of *SNR* of narrow band TEOAE components to the level of styrene exposure. Again, as for the studies described above, it is not possible to make a direct comparison with our findings as Triebig et al. (2009) examined possible changes of *SNR* with

exposure level but did not compared, as in our study, *SNR* of exposed subjects against that of control subjects.

TEOAEs of both cohorts exhibited the typical trend, described by a power law model, of shorter latencies for higher frequency components that characterizes linear OAE generation mechanisms. TEOAE latency is a measure of the roundtrip delay of the forward and backward travelling wave from the base of the cochlea to the reflection place and back, and reflects the mechanical properties of the basilar membrane, which are very sensitive to subtle OHC damage or dysfunction (Tognola et al., 1999; Moleti et al., 2005; Paglialonga et al., 2011). At the levels of styrene exposure investigated in this study, no significant changes were observed in the latency of TEOAE components of workers and controls of cohort 2. For cohort 1, a significant but of small amplitude change was observed in latency, as exposed subjects exhibited, on average, 0.49-ms-shorter latencies than the controls. Although significant at a statistical level, the observed latency change seems to be so marginal to represent a truly relevant alteration of the OAE generation mechanism.

Experimental findings of Yates and Withnell (1999) showed that in the healthy cochlea the nonlinear distortion mechanism is responsible for the production of intermodulation energy, which is originated by the interactions between each spectral component present in the eliciting click. Therefore, each frequency component of the OAE response could have been generated both by the response of its characteristic place along the BM and by the interaction between a pair of frequency components at almost any more apical place in the cochlea. As a consequence, any observed reduction in TEOAE energy at mid-to-high frequencies may be due both to a hearing loss at mid-to-high frequencies or to a decreased contribution of the intermodulation energy. As the *SNR* cannot discriminate between these two contributions (i.e.

that originated from the characteristic place along the BM and that associated to the intermodulation energy), we applied the bispectral analysis to investigate nonlinear cochlear mechanisms responsible for the generation of QFC components that may be associated to intermodulation energy.

AQFC was defined to quantify the contribution of QFC components to the signal energy in the TEOAE frequency bands. Current study revealed differences at a statistical level between the distributions of frequency bands showing QFC components in exposed and control subjects. As a general remark, TEOAEs of both exposed and control subjects clearly show the presence of QFC components in the range 500-4000 Hz, with the strongest contribution (i.e., the highest *AQFC* values) in the mid-to-high frequency range, nearly from 2000-2500 Hz to 4000 Hz and a significantly lower contribution in bands below 1600 Hz. Although exposed and control subjects exhibit the same ‘pattern’ of QFC component distribution that has been just described, local but significant differences at a statistical level were observed, as in exposed subjects the QFC component distribution appeared to be shifted towards lower frequencies than in control subjects. These findings might indicate that styrene (alone or in combination with noise) could influence the nonlinear distortion mechanism resulting in a shift of the intermodulation energy towards frequencies lower than in non-exposed subjects.

To conclude, the proposed advanced analysis revealed significant differences in the *SNR* and *AQFC* distribution of TEOAE components of exposed and control subjects and no systematic difference in the *LAT*. In particular, the analysis of *SNR* revealed significant reduction of energy in higher frequencies, but not in lower frequencies components. In contrast, the analysis of *AQFC* (and, to a lesser extent, *LAT*) revealed differences in low frequency components. Maybe, the *SNR* did not reveal any difference in the low frequency components

as this parameter is “contaminated” by the contribution of both the linear and nonlinear OAE components.

Our results suggest that low-level exposure to styrene may have induced a cochlear functionality modification as concerns both linear and nonlinear OAE generation mechanisms. As latency did not change, this might imply that the place of generation of OAE components is not affected by exposure to styrene at the levels considered in this study. Results obtained so far need to be confirmed in further analyses on a sample of larger size; also, it may be interesting in the future to investigate whether and how linear and nonlinear characteristics of TEOAEs elicited in styrene-exposed workers change during the day, in particular before and after the working shift.

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FIGURE CAPTIONS

FIG. 1 – Analysis of a representative control subject. (a) Recorded TEOAE (the two replicate recordings stored in buffers A and B are shown superimposed); (b) WT decomposition into time-frequency components. In each row $WT_A(\tau, f_k)$ and $WT_B(\tau, f_k)$ extracted from the two replicate recordings A and B are displayed and the corresponding SNR_k and LAT_k values are reported on the left side. For sake of clarity, only ten out of 48 WT frequency components have been reported (numbers on the right are the central frequencies of each component). (c) Frequency spectrum. (d) Contour plot of the magnitude of the bispectrum $\widehat{M}_3^x(f_1, f_2)$ in the principal domain region $[f_1 \geq 0, f_1 \geq f_2, f_1 + f_2 \leq \pi]$ and $AQFC$ values (black circles) obtained by the integration in the ten 1/3-octave frequency bands evidenced by the dashed counter-diagonals.

FIG. 2 – Mean SNR values ($\pm$ s.e.) in 1/3-octave bands from 500 to 4000 Hz of cohort 1 (a) and cohort 2 (b). Black symbols are for subjects exposed to styrene alone (S_E) and to styrene in combination with noise (NS_E); open symbols are for controls matched to the styrene group (S_C) and to the styrene and noise group (NS_C).

FIG. 3 – Mean LAT values ($\pm$ s.e.) in 1/3-octave bands of cohort 1 (a) and cohort 2 (b). Black symbols are for subjects exposed to styrene alone (S_E) and to styrene in combination with noise (NS_E); open symbols are for controls matched to the styrene group (S_C) and to the styrene and noise group (NS_C).

FIG. 4 – Mean $AQFC$ values ($\pm$ s.e.) in 1/3-octave bands of cohort 1 (a) and cohort 2 (b). Black symbols are for subjects exposed to styrene alone (S_E) and to styrene in combination with noise (NS_E); open symbols are for controls matched to the styrene group (S_C) and to the styrene and noise group (NS_C).

TABLE CAPTIONS

TABLE I. Individual group characteristics. Numbers within brackets are the min and max values observed for each measurement variable.

TABLE II. Results of the PTA examination in groups S_E and NS_E : symbol “X” means thresholds higher than those reported in the ISO7029 standard; symbol “O”: means thresholds within those reported in the ISO7029 standard.

TABLE III. F-values and, under brackets, degrees of freedom of the ANOVA tests applied to SNR and LAT values obtained from WT frequency components and to $AQFC$ values obtained with the bispectrum for the two examined cohorts. Main factors were: ‘group’ (exposed vs. control subjects), ‘frequency’ (ten 1/3-octave bands in the 500-4000 Hz range), and ‘age’ (for cohort 1, three age ranges: <40 yrs / from 40 to 44 yrs / ≥ 45 yrs; for cohort 2, two age ranges: <40 yrs / ≥ 40 yrs).

TABLE IV. *Post-hoc* test results on significant (group x frequency) interactions displayed in Table II. *Post-hoc* tests were performed on SNR and $AQFC$ values for groups S_E / S_C of cohort 1 and $AQFC$ values for groups NS_E / NS_C of cohort 2. Data reported under each column show only *post-hoc* significant ($p \leq 0.05$) comparisons of frequency means within groups. For example, “ f_1 vs f_3 - f_7 ” (see first column) means that the mean values observed at frequency band f_1 were significantly different from those at frequencies from f_3 to f_7 .

TABLE V. Mean values ($\pm$ s.e.) of SNR in age groups for exposed and control subjects of the two cohorts. The last row shows the results from the *post-hoc* comparisons of SNR between exposed and control subjects for each age group. Only *post-hoc* significant results ($p \leq 0.05$) are displayed.

Table I

		Cohort 1		Cohort 2	
		S _E (N = 9)	S _C (N = 9)	NS _E (N = 6)	NS _C (N = 6)
Age (yrs)		40.5 (29 – 48)	40.5 (29 – 48)	41.4 (33 – 53)	41.4 (33 – 53)
Sex	F	9	9	1	1
	M	-	-	5	5
Job tenure (yrs)		20 (5 – 26)	-	11 (3 – 27)	-
Styrene exposure TWA (mg/m ³)		47.3 (5.1 - 85.3)	-	<< 10	-
Noise exposure (dBA)		82.4 (80.7 – 83)	-	87.7 (86.7 – 88.0)	-

Table II

	# subject	ear	f (kHz)						
			0.5	1	2	3	4	6	8
S_E	1	R	X	O	O	O	O	O	O
		L	X	O	O	O	O	O	O
	2	R	O	O	O	O	O	O	O
		L	O	O	O	O	O	O	O
	3	R	O	O	O	O	O	O	O
		L	O	O	O	O	O	O	O
	4	R	O	O	O	O	X	O	O
		L	O	O	O	O	X	O	O
	5	R	X	O	O	O	O	X	O
		L	X	O	O	O	O	X	O
	6	R	O	O	O	O	O	O	O
		L	O	O	O	O	O	O	O
	7	R	X	O	O	O	O	O	O
		L	X	O	O	O	O	O	O
	8	R	O	O	O	O	O	O	O
		L	O	O	O	O	O	O	O
	9	R	X	O	O	O	X	O	O
		L	X	O	O	O	X	O	O
NS_E	1	R	O	O	O	O	O	O	O
		L	O	O	O	O	O	O	O
	2	R	X	O	O	O	O	O	O
		L	X	O	O	O	O	O	O
	3	R	X	O	O	O	O	O	O
		L	X	X	O	O	O	O	O
	4	R	O	O	O	O	O	O	O
		L	O	O	O	O	O	O	O
	5	R	X	O	O	O	O	O	O
		L	X	X	O	O	O	O	O
	6	R	X	O	O	O	O	O	O
		L	X	O	O	O	O	O	O

Table III

	Cohort 1			Cohort 2		
	<i>SNR</i>	<i>LAT</i>	<i>AQFC</i>	<i>SNR</i>	<i>LAT</i>	<i>AQFC</i>
Group	10.62** (1,359)	4.45* (1,211)	0.09 (1,359)	7.59** (1,239)	0.34 (1,91)	0.15 (1,239)
Frequency	14.81*** (9,359)	52.82*** (7,211)	63.25*** (9,359)	11.80*** (9,239)	21.12*** (4,91)	62.00*** (9,239)
Age	2.66 (2,359)	0.69 (2,211)	0.44 (2,359)	0.19 (1,239)	0.16 (1,91)	0.15 (1,239)
Group x Frequency	2.16* (9,359)	0.71 (7,211)	5.98*** (9,359)	1.59 (9,239)	1.77 (4,91)	9.16*** (9,239)
Group x Age	4.10* (2,359)	0.55 (2,211)	0.08 (2,359)	8.32** (1,239)	3.93 (1,91)	0.24 (1,239)
Group x Frequency x Age	0.29 (18,359)	0.45 (14,211)	0.32 (18,359)	0.33 (9,239)	0.70 (4,91)	1.31 (9,239)

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Table IV

Cohort 1				Cohort 2	
SNR		AQFC		AQFC	
S _E	S _C	S _E	S _C	NS _E	NS _C
f_1 vs $f_3 - f_7$	f_1 vs $f_3 - f_{10}$	$f_1 - f_4$ vs $f_6 - f_{10}$	$f_1 - f_5$ vs $f_7 - f_{10}$	$f_1 - f_4$ vs $f_6 - f_{10}$	$f_1 - f_3$ vs $f_5 - f_{10}$
f_2 vs $f_4 - f_5$	f_2 vs $f_4 - f_8$	f_3 vs $f_7 - f_9$	$f_6 - f_7$ vs $f_8 - f_{10}$	f_3 vs $f_7 - f_{10}$	f_4 vs $f_6 - f_9$
f_4 vs $f_8 - f_{10}$				f_6 vs $f_8 - f_9$	f_5 vs $f_7 - f_8$
f_5 vs $f_9 - f_{10}$					f_6 vs f_8
					f_7 vs f_{10}
					f_8 vs $f_9 - f_{10}$
$f_1 = 500$ Hz; $f_2 = 625$ Hz; $f_3 = 800$ Hz; $f_4 = 1000$ Hz; $f_5 = 1250$ Hz; $f_6 = 1600$ Hz; $f_7 = 2000$ Hz; $f_8 = 2500$ Hz; $f_9 = 3200$ Hz; $f_{10} = 4000$ Hz.					

Table V

	Cohort 1			Cohort 2	
	SNR (dB)			SNR (dB)	
	< 40 yrs	40 - 44 yrs	≥ 45 yrs	< 40 yrs	≥ 40 yrs
Exposed	7.8 (0.6)	7.9 (0.8)	8.3 (1.0)	7.7(3.4)	4.9 (3.0)
Controls	12.6 (0.6)	9.1 (0.4)	8.8 (0.8)	7.6 (2.9)	9.6 (3.0)
$p \leq 0.05$	Exp vs Contr			Exp vs Contr	

Figure 1

Figure 2

Figure 3

Figure 4