Oxidative stress in adolescent passive smokers living in urban and rural environments

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(Article begins on next page)
Abstract: Purpose of this study was to evaluate and compare the oxidative stress status - a pre condition often resulting from imbalance between the production of oxidants and antioxidants in the body - among a group of adolescents. This work was conducted through the measurement of a specific oxidative stress biomarker: the urinary 15-F2t-isoprostane (15-F2t-isoP) in 168 adolescents passively smokers: 110 urbanized and 58 living in the countryside. A general linear model (GLM) analysis was performed in order to evaluate the significance of two factors in the biosynthesis of 15-F2t-isoP measured with ELISA technique: residence and tobacco smoke exposure, measured with urinary cotinine. Formaldehyde (FA) concentration in air was also evaluated as a primary confounding factor in oxidative stress. No significant differences in FA contamination between the two sites were found. Conversely, direct relationship between oxidative stress status and residence of adolescents was found: oxidative stress level was 31% higher for adolescents living in urban site than for those living in the countryside area. Tobacco smoke exposure proved to play an important role in the distribution of 15-F2t-isoP levels (p < 0.0001). Lastly, an inversely proportional relationship was found between the age of adolescents and 15-F2t-isoP (p < 0.0001).

The biological responses evaluated in this study suggested that even a modest environmental level of pollutants may affect the exposure risk and should possibly be taken into account to plan primary prevention actions. Future investigations will explore the effect of other pollutants, including the particulate matter.

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January 11th, 2013

Dear Editor of *International Journal of Hygiene and Environmental Health*:

Please find enclosed the manuscript “Oxidative stress in adolescent passive smokers living in urban and rural environments” by R. Bono et al. In my view, the relevance of the results summarized in the manuscript is two-fold. Firstly, the evidence that passive tobacco smoke exposure causes oxidative stress in adolescents, independently from the urbanization level. Secondly, oxidative stress increases according to the level of urbanization of the adolescents, independently from their passive tobacco smoke exposure. Thus, data may hence represent a platform for designing a protective grid for prevention to tobacco smoke exposure and to urban pollution.

Some information about the results presented:

- The new version of the manuscript has been corrected according to author instructions.
- The manuscript is an original work, has not been previously published in whole or in part, and is not under consideration for publication elsewhere;
- The voluntary participation of all the human subjects did not occur until after informed consent was obtained;
- All authors have disclosed any potential competing interest regarding the submitted article and the nature of those interests;
- All the authors have read the manuscript, agree that the work is ready for submission to Journal of Adolescent Health, and accept responsibility for the manuscript’s contents.

Hoping that the manuscript may fulfil the scientific standards of *International Journal of Hygiene and Environmental Health*, my best regards.

Roberto Bono, Ph. D.

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Abstract

Purpose of this study was to evaluate and compare the oxidative stress status - a pre condition often resulting from imbalance between the production of oxidants and antioxidants in the body - among a group of adolescents. This work was conducted through the measurement of a specific oxidative stress biomarker: the urinary 15-F2t-isoprostane (15-F2t-isoP) in 168 adolescents passively smokers: 110 urbanized and 58 living in the countryside. A general linear model (GLM) analysis was performed in order to evaluate the significance of two factors in the biosynthesis of 15-F2t-isoP measured with ELISA technique: residence and tobacco smoke exposure, measured with urinary cotinine. Formaldehyde (FA) concentration in air was also evaluated as a primary confounding factor in oxidative stress. No significant differences in FA contamination between the two sites were found. Conversely, direct relationship between oxidative stress status and residence of adolescents was found: oxidative stress level was 31% higher for adolescents living in urban site than for those living in the countryside area. Tobacco smoke exposure proved to play an important role in the distribution of 15-F2t-isoP levels (p < 0.0001). Lastly, an inversely proportional relationship was found between the age of adolescents and 15-F2t-isoP (p < 0.0001).

The biological responses evaluated in this study suggested that even a modest environmental level of pollutants may affect the exposure risk and should possibly be taken into account to plan primary prevention actions. Future investigations will explore the effect of other pollutants, including the particulate matter.

Key words

Oxidative stress, 15-F2t-isoprostane, passive tobacco smoke, urban pollution, adolescents
Introduction

Indoor and outdoor air pollution, hazardous chemicals, noise, food and water contaminants are factors possibly associated with environment-related health outcomes, including respiratory diseases, allergies and asthma, cardiovascular diseases, neurological effects, reproductive and developmental disorders, and cancer. Among the environmental factors, urban outdoor air pollution, partly generated by car exhaust, has become a problem of growing international interest (Bono et al., 2010a; Cohen et al., 2005; Tzivian, 2011).

Air pollution may present various physical, chemical, mutagenic and toxicological properties, according to geographical area and human socio-economic activities (Traversi et al., 2008). The resulting impact on human health may evolve with different characteristics and intensity levels (Gomes et al., 2012; Plummer et al., 2012). Epidemiological studies performed in metropolitan areas revealed that the exposure to urban air pollution is a significant factor in the increasing prevalence of many diseases and mortality, even if its mechanisms of action remains partially unclear (Brauner et al., 2007; Brunekreef, 2007). In urban areas, prevalent contribution to air pollution arises from motor vehicle emissions (Gomes et al., 2012). Thus, many researchers focused their studies on exposure assessment and measurement of primary biological and/or adverse health effects on citizens exposed to traffic-related pollutants (Bind et al., 2012; Gan et al., 2012).

Recently, many scientific evidences attributed to air pollution and vehicle exhaust emissions the effect to modulate the body’s redox system through an increase of pro-oxidant species and a decrease of antioxidant molecules (Ghio et al., 2012; Yang and Omaye, 2009). This condition, defined as oxidative stress, is connected with several DNA lesions, including modifications of bases, which are considered potential causes of cancer (Ghio et al., 2012; Loeb, 2001).

Oxidative stress can be induced by outdoor and indoor environments (residential, public or occupational) (Bono et al., 2010b). The indoor environments, particularly the sites where people smoke or have smoked, are often characterized by the highest levels of pollutants that induce oxidative stress (Fuselli et al., 2010). Accordingly, oxidative stress plays a crucial role in the
inflammatory response to tobacco smoke (Doruk et al., 2011), frequently in co-exposure with airborne ultrafine particles (Mo et al., 2012). Tobacco smoke is a complex mixture of oxidizing compounds, capable to promote numerous biological damages, such as lipid peroxidation (Kalra et al., 1991; Morrow et al., 1995; Scherer, 2005), protein and thiol oxidation (Frei et al., 1991; Reznick et al., 1992), and oxidation of DNA (Park et al., 1998). The combustion-derived nanoparticles (CDNPs), present in tobacco smoke and all environmental contests, produce oxidative stress, inflammation and lung cancer. CDNPs can be redistributed to other organs, after pulmonary deposition (Donaldson et al., 2005). The knowledge of how exogenous and endogenous oxidants interact with molecules in the cells, tissues, and the epithelial lining fluid (ELF) of the lung is crucial for planning the most suitable prevention strategies.

F2-isoprostanes are specific products of lipid peroxidation and their metabolites were evaluated in vivo as potential biomarkers of oxidative stress status (Roberts and Morrow, 2000). F2-isoprostanes are a family of Prostaglandin (PG) F2α isomers, described as products of non-cycloxygenase after oxidative modifications of arachidonic acid, that resulted from free-radical attack of cell membrane phospholipids or circulating low density lipids (LDLs) (Lynch et al., 1994; Morrow et al., 1990). Thus, F2-isoprostanes, a chemically stable group of bioactive compounds, appear to play a role in acute and sub-clinical chronic inflammations (Basu et al., 2009) and are utilized as non-invasive markers of airways inflammation (Basu, 2008) and asthma (Wedes et al., 2009). They may describe the possible role of some exogenous factors in the expression of oxidative stress in selected populations. They can also be implicated in a large number of human diseases, even if a clear correlation between disease and oxidative stress is far from being proven for most pathological conditions (Giustarini et al., 2009).

Purpose of this study was to clarify the role of the exposure to tobacco smoke and air pollution in the biosynthesis of 15-F2t-isoprostane (15-F2t-IsoP) (Romanazzi et al.) within two population of adolescents, living in areas of the Piedmont Region (North-Western Italy) characterized by different geographical conditions and levels of urbanization.

4
Methods

Sampling Sites. Two sampling sites were selected taking into account different urbanization, anthropization and vehicular traffic conditions. Chivasso is an urbanized city with about 26,000 inhabitants (514 inhabitants/km²), located at 180 meters a.s.l. close to Torino, the capital of Piedmont Region (900,000 inhabitants). Casalborgone (200 meters a.s.l.) is a rural site, 12 km away from Chivasso, and populated by 1850 inhabitants (92 inhabitants/Km²). (Sources by Piedmont Region, 2011) (Figure 1).

Epidemiological sample. All adolescents (N=168) involved in the present study were volunteers who attended two schools located in Chivasso (N=110) and Casalborgone (N=58), respectively. Since the subjects were underage, during a public meeting, parents and teachers were informed on the objective of this study and to parents were asked to sign a written informed consent. Thus, the participation of all the human subjects did not occur until after informed consent was obtained. Sampling was carried out from March to April 2011, involving one class per day, according to a pre-established timetable. From each student, the following items were collected: (1) a questionnaire, gathering information about general features, (2) a urine sample for the quantification of urinary cotinine, 15-F₂IsoP and creatinine (CREA), (3) spirometry data to evaluate respiratory health and vital capacity.

Questionnaire. A questionnaire was administered to each subject during the school time, to collect information concerning individual and clinical features, such as age, gender, residence, hobbies, therapies, and parent’ smoking habits.

Urinary cotinine. Urinary cotinine was measured in order to consider the possible role played by passive tobacco smoke in the onset of an oxidative stress status. An aliquot of fresh urine was collected in the early morning and approximately at the same time from each volunteers, and stored at -80 °C prior to analysis, performed within 20 working days. 10 ml of urine was transferred into a glass tube and 4 g of NaCl, 500 µl of NaOH (5M) and 10 µl of cotinine-d₃ (internal standard) were
added. Subsequently, for two times, 2 ml of trichloromethane (CHCl₃) were added to the sample to perform liquid-liquid extraction which was carried out in a shaking wheel for 15 minutes. Sample was then centrifuged for 10 min at 1000 g and the resulting organic phase was collected in a new glass tube and evaporated to dryness in a rotary evaporator at room temperature. The dry residue was reconstituted in 200 µl of CHCl₃ and transferred into a conical vial for GC-MS determination. All the details of this last instrumental procedure were reported in a previous paper (Bono et al., 2012).

The cotinine calibration curve was built by fortifying a blank urine pool of non-smoking subjects, to obtain a concentration range from 0.02 µg/ml to 2 µg/ml. the fortified urine was extracted as for the samples. The limit of detection (LOD) was calculated as the concentration of the analyte that gives a signal equal to the average background of the blank (S_blank) plus three times its standard deviation (LOD = S_blank + 3S_blank), while the limit of quantification (LOQ) was estimated as twice of the LOD value. LOD and LOQ were respectively 0.01 µg/ml and 0.02 µg/ml. Coefficients of variation (CV%) calculated to test repeatability were below 5% for both cotinine and the internal standard.

**Urinary Isoprostane.** 15-F₂t-IsoP in urine was measured by ELISA technique performed with a specific microplate kit (Oxford, MI, USA), according to manufacturer’s instructions. All the details of this procedure were reported in a previous paper (Romanazzi et al.). Dilution 1:4 was adopted to achieve better accuracy in the competitive ELISA method.

**Creatinine quantification.** An aliquot of fresh urine was used to measure the concentration of urinary creatinine (crea) by the kinetic Jaffé procedure (Bartels and Cikes, 1969) so as to normalize the excretion rate of urinary cotinine and 15-F₂t-IsoP.

**Spirometry.** For each subject, maximal expiratory flow-volume curves were obtained in standing position, wearing a noseclip and breathing into a Stead-Wills spirometer. The instrument was calibrated with a 3L syringe daily. Spirometry curves were collected three times until they were repeatable within a 5% experimental error. Values were corrected for BTPS (Body Temperature Pressure Standard). Measured spirometric parameters included best forced vital capacity (FVC),
forced expiratory volume in one second (FEV\textsubscript{1}) and maximal expiratory flows at peack, 50%, 25% (PEF, MEF\textsubscript{50}, MEF\textsubscript{25}) (Miller et al., 2005).  

Formaldehyde sampling and analysis  

Formaldehyde determinations were carried out daily during three months of sampling (March, April, May 2011), in two sites close to the adolescent’ schools. For each sampling site, a sampling line working at 1.0 l/min sampling speed during 24 hours/day and equipped with a adsorbent vial containing silica gel coated with 2,4-dinitrophenylhydrazine (DNPH) was used. The reaction of DNPH with FA yielded 2,4-dinitrophenylhydrazone which was subsequently quantified by a GC-MS method, as described elsewhere [33].  

Statistical analysis. The analysis was performed by means of Stata 12 Statistical Package (StataCorp LP, Lakeway Drive, TX, USA). Appropriate linear transformation was applied on data whenever suggested by distributional diagnostic plots (symmetry plot, quantile plot) and descriptive statistic inspection (looking at variance stability among categories). To alleviate multivariate heteroscedasticity, a Box-Cox power transformation on the dependent variable was applied to the data by means of maximum likelihood estimates of the parameters. To compare the values among groups, a median test (a nonparametric K-sample test on the equality of medians) was performed, checking the null hypothesis that the K samples were drawn from populations with the same median.  

The relationship of 15\textsubscript{2t}-IsoP with the other relevant variables was studied by means of the general linear model (GLM) analysis, that considers 15-F\textsubscript{2t}-IsoP as the dependent variable, while respiratory symptoms recorded by the questionnaire (cough, colds, catarrh, whistles, shortness of breath, lung disease, bronchitis, rhinitis and allergies), sampling sites, gender, Box-Cox transformed level of cotinine, age (expressed as days since the birth date divided by 365.25), weight (Kg), height (meters) and B.M.I. (weight/height(m)\textsuperscript{2}), were the independent variables. To assess the relationship between lung function parameters (FEV\textsubscript{1}, FEF 25-75 and FEF50) and 15-F\textsubscript{2t}-IsoP, a different linear model (GLM) analysis was performed that considers 15-F\textsubscript{2t}-IsoP as the dependent
variable, and gender, Box-Cox transformed single lung function parameter and level of cotinine, age (years), weight (Kg), height (meters) and B.M.I. (weight/height(m)²), as independent variables.

The link function for GLM was selected following the Box-Cox transformation. A \( p \) value of ≤0.05 (two-tailed) was considered significant for all tests. All the variables that proved non-significant at 5% were excluded with a step-wise backward removal procedure.

**Results**

The main characteristics of the population examined are presented in Table 1. Subjects were grouped according to their residence municipality, which in turn coincides with the school location: Chivasso (urban site) and Casalborgone (rural site), respectively. For each group of students, numerosity, gender, averaged age, weight, height, and information about passive smoke exposure are reported.

Normalized 15-F₂t IsoP values for the investigated populations are given in Table 2. On average, 15-F₂t IsoP values recorded in Chivasso are significantly higher than those found in Casalborgone (\( p = 0.03 \)). This outcome highlights an unspecific “urban factor” that promotes a higher level of oxidative stress in the adolescent subjects living and attending the school in the urban site.

The Box-Cox estimate and variables inspection suggested to apply a natural log-transformation on urinary cotinine concentrations and to use log-function as a link in the GLM: the other covariates and factors were not transformed. No significant relationship was detected between 15-F₂t-IsoP and respiratory symptoms, gender, weight, and height (\( p > 0.10 \)). Table 3 shows GLM outcomes considering log-urinary cotinine levels, sampling site and age.

A positive relationship was found between urinary 15-F₂t-IsoP and cotinine levels and urbanization of the sampling sites; while a negative relationship was found with respect to the adolescents’ age. Multivariate analysis shows a positive effect of log-cotinine concentration on 15F₂t-IsoP level (\( p < 0.0001 \)). In particular, a 27% increment of 15F₂t-IsoP is observed for each one-unit-increment of
log-cotinine (2.71828 in natural scale). Thus, it can be hypothesized that passive tobacco smoke exposure causes oxidative stress in the adolescent subjects, independently from the urbanization level (urban or rural site) and the subject’s age (Figure 2A). Similarly, GLM analysis shows significant higher 15-F$_{2t}$-IsoP levels for the Chivasso population ($p < 0.0001$), i.e., the mean level of 15-F$_{2t}$-IsoP 31% is higher than for the Casalborgone sample population, independently from passive smoke exposure (Figure 2B). Finally, the GLM analysis shows a 15F$_{2t}$-IsoP decrease as a function of the increasing subjects’ age ($p < 0.001$) with a 19% decreases every 12 months of age (Figure 2C). Lung function parameters are not significantly related (5% level) to 15-F$_{2t}$-IsoP, after controlling for age, sampling site and cotinine level (data not shown). Finally, mean concentrations of airborne FA (expressed as µg/m$^3$) were simultaneously measured outside the urban and rural schools. No evident differences between the two sites (t-test) were found. The recorded values are also comparable with those reported in the literature (NTP, 2011).

**Discussion and conclusion**

The specific purpose of this study was to assess the level of oxidative stress, as estimated from the urinary 15-F$_{2t}$-IsoP specific biomarker, as a function of various individual parameters, using two populations of adolescents respectively living in an urbanized site and a countryside area. Among these parameters, the passive exposure to tobacco smoke was also quantified for each subject by means of its urinary cotinine.

Adolescents were used as a target population for investigating the effects of different environmental conditions on the onset of an oxidative stress status, since they have on average lower mobility and a simpler life-style than adults, so that most of their time is spent within resident domestic and scholar indoor ambients.

The principal result of this study is the observation of positive and direct relationship between urbanization level and oxidative stress status, which appears to be independent from passive smoke exposure. In the urban site of Chivasso, detected urinary 15-F$_{2t}$-IsoP mean levels were 31% higher
than those found for the population living in the rural site of Casalborgone. This result could be partially explained by a generic “urban air pollution” factor which is definitely higher in Chivasso. However, air-FA, identified \textit{a priori} as a possible marker of air pollution, failed to show significantly higher concentrations in the urbanized area than in the rural site. The similar of air-FA concentration in the two sites could be attributed to the complex and multiple generation mechanisms of this pollutant, ranging from primary origins, chiefly occurring in the urban environments, to secondary (photochemical) origin in the case of rural sites. Probably, determinations of individual exposure to air-FA and other airborne pollutants by using personal samplers may provide more accurate and highly sensitive observations and, therefore, could be performed in future investigations.

The second important result gained in this study is the evidence of the role played in the 15-F$_2$t-IsoP formation by tobacco smoke exposure, independently from the urbanization level. This means that an exposure, although passive, to about 4000 chemicals present in tobacco smoke acts as an inductor of significant oxidative stress. Thus, both independent factors investigated in this study, namely urbanization level and passive tobacco smoke, were found to have a powerful role in the induction of oxidative stress status detected by 15-F$_2$t-IsoP.

The students’ age also appears to play a role in the formation of 15-F$_2$t-IsoP, although a very limited age range is deliberately represented in the investigated population (12, 13 and 14 years old). Surprisingly, age and 15-F$_2$t-IsoP appear to be inversely correlated, whereas, in previous studies, an increase of plasma free and total (free plus esterified) 15-F$_2$t-IsoP levels was found with the patients’ age (Cruz et al., 2009; Ward et al., 2005). On the other hand, previous studies did not consider homogeneous populations of healthy adolescents, as in the present case. Thus, further investigations expanding the studied age range appear to be necessary.

Finally, others factors taken into account, including the non specific respiratory symptoms (as collected by questionnaire) and spirometric measurements, proved not to be associated with 15-F$_2$t-IsoP in urine, at least in the age range considered in this study. This could be related to the
relatively small concentration of the biomarker. However, although a cross-sectional effect cannot be demonstrated in our study, a possible long-term effect on lung function should not be excluded, as demonstrated for environmental pollutants in other sites (Arossa et al., 1987).

Remarkably, no 15-F_{2t}-IsoP outliers were detected in the studied healthy population. Therefore, the detection of such a sensitive biological response as a consequence of limited differences of environmental pollution could provide new and useful knowledge for the appraisal of preventive strategies, particularly for young subjects, which are known to be more sensitive, since they spend most of their time in indoor environments (i.e. school and home), and they have a respiratory system not immunologically fully mature yet (Neri et al., 2006). Thus, we intend to extend this study to a larger number of subjects and sites, presenting different environmental characteristics. To better explain the biological effects, we plan to consider a higher number of air pollutants and, in particular, the ultrafine particulate as another important “urban air factor”, possibly involved in the onset of oxidative stress of environmental origin.

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Conflict of interest: The authors have no conflicts of interest to disclose
References


Figure 2

A) 

B) 

C)
Figure 1: The two sampling sites: Chivasso (urban site) and Casalborgone (rural site) compared to Torino.

Figure 2: A) Added-variable plot of log (15F2t-IsoP) levels by log (cotinine), adjusted by age and “sampling site”. r = multivariate residual given age and sampling site. B) Added-variable plot of log (15F2t-IsoP) levels by the two sampling sites, adjusted by age and log(cotinine). r = multivariate residual given age and log (cotinine). C) Added-variable plot of log(15F2t-IsoP) by age, adjusted by “sampling site” and “log (cotinine)”. 
<table>
<thead>
<tr>
<th></th>
<th>CHIVASSO</th>
<th>CASALBORGONE</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N.</strong></td>
<td>110</td>
<td>58</td>
<td>168</td>
</tr>
<tr>
<td><strong>GENDER</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male n (%)</td>
<td>59 (53%)</td>
<td>26 (45%)</td>
<td>85 (50.5%)</td>
</tr>
<tr>
<td>Female N (%)</td>
<td>51 (47%)</td>
<td>32 (55%)</td>
<td>83 (49.5%)</td>
</tr>
<tr>
<td><strong>AGE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>years ± s.d.</td>
<td>12.7 ± 0.8</td>
<td>12.5 ± 0.6</td>
<td>12.6 ± 0.8</td>
</tr>
<tr>
<td><strong>WEIGHT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kg ± s.d.</td>
<td>47.3 ± 12.3</td>
<td>47.7 ± 12.2</td>
<td>47.5 ± 12.2</td>
</tr>
<tr>
<td><strong>HEIGHT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cm ± s.d.</td>
<td>154.7 ± 8.9</td>
<td>153.0 ± 9.8</td>
<td>154.2 ± 9.2</td>
</tr>
<tr>
<td><strong>SMOKE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active smokers n. (%)</td>
<td>1 (0.9%)</td>
<td>0</td>
<td>1 (0.5%)</td>
</tr>
<tr>
<td>Passively exposed n. (%)</td>
<td>50 (45.5%)</td>
<td>23 (60.3%)</td>
<td>73 (43.5%)</td>
</tr>
<tr>
<td>not exposed n. (%)</td>
<td>59 (53.6%)</td>
<td>35 (39.7%)</td>
<td>94 (56.0%)</td>
</tr>
</tbody>
</table>

Table 1. Epidemiological characteristics and exposure to tobacco smoke of the whole populations grouped according to their residence and school.
<table>
<thead>
<tr>
<th>SAMPLING SITE</th>
<th>MEAN ± S.D.</th>
<th>MIN</th>
<th>MAX</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chivasso (urban)</td>
<td>5,8 ± 5,1</td>
<td>1,2</td>
<td>39,8</td>
<td>0,03</td>
</tr>
<tr>
<td>Casalborgone (rural)</td>
<td>4,8 ± 2,9</td>
<td>1,5</td>
<td>14,7</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5,5 ± 4,5</td>
<td>1,2</td>
<td>39,8</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. $15$-$F_{2\alpha}$ IsoP values in the two group of students attended the two schools.
Table 3. GLM analysis results using $^{15}$F$_{2}$-IsoP as dependent variable with link log and normal distribution assumption (non significant effects at 5% level are not reported).