石墨烯联合磷酸三苯酯(TPP)胁迫紫贻贝(*Mytilus galloprovincialis*)的生理生化响应

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摘要 石墨烯纳米材料具有优良的吸附性能, 进入环境后容易与其他污染物发生相互作用, 进而影响其环境行为和毒理学效应。磷酸三苯酯(triphenyl phosphate, TPP)在环渤海中具有广泛的分布, 且分子中含有多个苯环, 易与石墨烯发生相互作用。为了研究石墨烯和TPP对海洋贝类生理响应的影响, 采用指示生物紫贻贝(*Mytilus galloprovincialis*)进行了单一及联合暴露实验。通过对滤水率、耗氧率和排氨率的检测, 分析了暴露物对紫贻贝生理代谢的影响; 制作石蜡切片观察鳃和肝胰腺组织的结构变化; 采用酶活试剂盒对肝胰腺组织中的抗氧化酶活性进行检测。结果表明, 石墨烯和TPP单独暴露, 均可对紫贻贝产生氧化胁迫和组织损伤。石墨烯和TPP联合暴露对紫贻贝的氧化胁迫和组织损伤程度, 低于石墨烯单独暴露组。石墨烯对紫贻贝的耗氧率和排氨率均有显著影响($P<0.05$)。与对照组相比, 石墨烯显著抑制紫贻贝的呼吸, 增加紫贻贝的排氨率。TPP暴露后显著增加紫贻贝的排氨率。联合暴露后可降低由石墨烯引起的呼吸抑制, 且升高氧氮比(O:N), 说明联合暴露对机体造成的压力低于石墨烯单独暴露。综合生物标志物指数(IBR)结果显示, 联合暴露对紫贻贝产生的胁迫压力低于石墨烯和TPP分别单独暴露。本研究结果可为预测海洋环境污染对贝类以及海洋生态系统产生的影响提供参考。

关键词 紫贻贝, 石墨烯, 磷酸三苯酯, 联合作用, 生理响应

工程纳米材料(NPs)产量的增加引起了人们对其潜在生物影响和生态效应的关注。石墨烯被认为是目前生态毒理学研究中使用最频繁、研究最广泛的NPs之一^[1]。由于石墨烯的优异性能, 使其在许多领域都有应用前景, 例如半导体^[2]、纳米复合材料^[3]和生物医药^[4]等。由于检测方法的局限性, 关于石墨烯类纳米材料的环境浓度报道较少。氧化石墨烯(GO)是石墨烯的重要衍生物之一, 其表面经过功能化修饰, 连接含氧活性基团, 为石墨烯的氧化物形式^[5]。Hu等人^[6]研究发现, GO

的环境相关浓度为1 mg/L。文献^[7]报道, 预测水环境中GO的浓度约为1~1000 $\mu\text{g/L}$ 。石墨烯可通过多种途径释放到河口和近岸海域中, 对海洋生物健康构成的威胁不容忽视^[8,9]。有研究报道了石墨烯类纳米材料对水生生态系统和海洋无脊椎动物的潜在影响^[10,11]。例如, 在亚致死浓度下(0~25 mg/L), GO和还原性GO-聚乙烯吡咯烷酮(rGO-PVP)均可增加紫贻贝血细胞中ROS的生成, 并且GO在25 mg/L的最高亚致死浓度下显著增加细胞内ROS的生成^[12]。

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由于纳米材料的比表面积和孔径较大, 纳米材料易与其他污染物相互作用, 从而影响其环境行为和毒理效应^[13,14]. 富勒烯纳米颗粒(C_{60})可作为重金属镉(Cd)和锌(Zn)的载体, 促进Cd和Zn向水生生物的转移^[15]. 二氧化钛纳米颗粒(nano-TiO₂)已被证实会增强铅(Pb)对斑马鱼中枢神经系统的发育毒性^[16]. 二氧化钛纳米颗粒可增强Cd在嗜热纤毛虫(*Tetrahymena thermophila*)体内的生物积累, 并具有明显的亚细胞分布^[17]. 然而, GO和铜(Cu)对斜生栅藻(*Scenedesmus obliquus*)有显著的拮抗作用, GO可显著降低铜Cu的生态毒性^[6]. 上述研究表明, 纳米材料可通过改变其他污染物的生物利用度对其毒性产生影响. 虽然围绕石墨烯类材料对水生生物的风险性开展了研究, 但石墨烯与其他污染物产生的联合影响(例如有机磷阻燃剂)仍需深入关注.

生理参数反映了生物体内与能量平衡有关的过程和应对环境胁迫的生理可塑性, 是评价环境压力对海洋双壳贝类影响的可靠指标^[18,19]. 摄食和代谢作为生理参数研究的主要内容, 受到学术界的普遍关注. 海洋水生动物的运动及能量转化依靠生物体内的有氧代谢进行, 因而水中氧气的消耗常作为衡量动物能量消耗的一个指标. 海洋水生动物对蛋白质代谢的最终产物为氨态的氮, 因此氨态氮可作为研究生物代谢的常用指标^[20]. 耗氧率、排氨率的大小及变化规律在很大程度上反映了机体的真实代谢水平及变化规律, 若代谢产生紊乱, 会对生物体的生长和健康等过程产生不利影响^[21].

目前, 关于海洋水生动物生理功能的研究已有大量报道, 如八面体纳米Cu₂O可导致大型蚤(*Daphnia magna*)肠道内产生ROS, 对大型蚤产生显著的氧化胁迫和膜损伤^[22]. 王有基等人^[23]综述了近年来海洋酸化对贝类早期发育、钙化、免疫和繁殖等生理生态的影响. 高浓度(50~100 mg/L)多壁碳纳米管(MWCNTs)抑制铜绿微囊藻(*Microcystis aeruginosa*)的生长, 导致藻细胞生命活力下降, 过氧化物歧化酶(SOD)、过氧化物酶(POD)、过氧化氢酶(CAT)活性逐渐降低^[24]. 磷酸三苯酯(triphenyl phosphate, TPP)在各介质中广泛分布, 具有潜在的内分泌干扰作用^[25]、神经毒性^[26]和发育毒性^[27]. 随着石墨烯和TPP需求量和生产量的日益增长^[1,27], 二者可能同时存在于环境中, 目前的研究大多只考虑单因素暴露, 而对于两者之间的相互作用认识不足. 因此亟待深入研究石墨烯和TPP的联合毒理效应及作用机制.

海洋双壳类是评价污染物对水生生物群落影响的重要生物, 可作为监测海洋污染的目标群体^[28,29]. 作为滤食性动物, 紫贻贝具有较好的污染物积累和耐受能力^[30]. 本研究以紫贻贝为目标生物, 评估石墨烯和TPP暴露情况下紫贻贝的生理响应(清除率、呼吸率、氨排泄率和氧氮比O:N)、组织损伤和生化响应指标, 包括谷胱甘肽S-转移酶(GST)、SOD、谷胱甘肽过氧化物酶(GPx)、CAT的活性和谷胱甘肽含量(GSH). 在此基础上, 结合生物标志物综合响应指数(IBR)建立了石墨烯联合TPP胁迫暴露后紫贻贝的生理生化响应评估体系.

1 材料与方法

(i) 试剂. 石墨烯(1.0 mg/mL)购于美国Sigma-Aldrich公司, TPP(纯度99%)购于德国Dr. Ehrenstorfer公司. 实验用水经过Cascada LS超纯水机(美国Pall公司)纯化至电阻率大于18 MΩ cm. 为确定石墨烯的形态和尺寸, 采用扫描电子显微镜(SEM)和原子力显微镜(AFM)表征石墨烯. 实验前将石墨烯超声震荡15 min, 确保石墨烯纳米颗粒分散均匀.

(ii) 实验设计. 实验用紫贻贝购于烟台烟大市场, 体长4~5 cm. 使用过滤海水(500目筛网过滤)暂养紫贻贝7 d, 每天定时投喂等鞭金藻 25×10^4 cells/(mL d), 喂食2 h后换水. GO的环境相关浓度为1 mg/L^[5]. 对于海洋无脊椎动物, TPP的半数有效浓度(EC₅₀)为0.26~2.0 mg/L^[31]. 我们前期采用电化学方法研究石墨烯和TPP与模拟生物膜间的相互作用发现, 0.5 mg/L暴露浓度下石墨烯和TPP显著影响细胞膜的通透性, 破坏模拟生物膜的完整性^[32]. 参照研究报道和我们的前期研究结果^[6,31,32], 本研究采用的石墨烯和TPP暴露浓度均为0.5 mg/L. 紫贻贝在驯养和暴露期间的养殖环境一致, 均采用过滤海水, 盐度为32, 海水温度为18°C. 驯养和实验期间, 每天定时投喂等鞭金藻, 喂食2 h后定时更换海水. 每次更换海水后重新加入相应比例的石墨烯和TPP母液, 使各暴露组中的石墨烯和TPP达到预定浓度. 暂养结束后开始正式暴露实验, 共分为5组: 海水对照组(对照)、溶剂对照组(DMSO)、0.5 mg/L石墨烯暴露组(石墨烯)、0.5 mg/L TPP暴露组(TPP)、0.5 mg/L石墨烯与0.5 mg/L TPP联合暴露组(石墨烯+TPP), 每组20个个体. 暴露14 d后, 进行各项指标检测.

(iii) 生理指标. 根据以下公式计算滤水率:

$$CR = (V/Nt)\ln(C_0/C_t), \quad (1)$$

式中, CR为单位体重的滤水率(L/(g h)), V 为暴露体积(L), N 为实验贝个数, t 为实验时间(h), 本实验为2 h. C_0 , C_t 分别为实验开始及 t 时间后饵料浓度($\times 10^4$ cells/mL), 饵料浓度用显微镜计数测定.

根据下式计算摄食率:

$$IR = CR \times C_0, \quad (2)$$

式中, IR为单位体重的摄食率(cells/(g h)).

溶解氧(DO)采用Winkler碘量法, 氨氮(NH_4^+-N)采用次溴酸钠氧化法. 根据下式计算耗氧率:

$$OR = (D_0V_0 - D_tV_t) / (T \times N), \quad (3)$$

式中, OR为单位体重的耗氧率($\text{mg O}_2/(\text{g h})$); D_0 和 D_t 分别为实验开始和实验结束时呼吸瓶中溶氧的含量($\text{mg O}_2/\text{L}$); V_0 和 V_t 分别为实验开始和试验结束时呼吸瓶中实验用水的体积(L); T 为实验时间(h), 本实验为2 h; N 为实验生物的个数.

根据下式计算排氨率:

$$NR = (N_{et} - N_{e0}) - (N_{ct} - N_{c0}) \times V / (T \times t), \quad (4)$$

式中, NR为单位体重的排氨率($\text{mg NH}_4\text{-N}/(\text{g h})$); N_{et} 和 N_{e0} 为平行组实验结束和开始时水中 NH_4^+-N 含量(mg/L); N_{ct} 和 N_{c0} 为空白对照组实验结束和开始时水中 NH_4^+-N 含量(mg/L); V 为呼吸瓶中水的体积(L), T 为贝个数, t 为实验时间(h), 本实验为2 h.

(iv) 组织病理学. 暴露14 d后, 冰上获取紫贻贝的肝胰腺和鳃组织, 用Bouin固定液固定. 固定24 h后, 将组织在渐进的乙醇系列中进行脱水(70%、85%、95%和100%)并包埋于石蜡中. 利用石蜡切片机获取厚度为

4 μm 的组织切片, 固定于载玻片上, 采用苏木精和伊红染色, 最后在200倍光学显微镜(Olympus BX61, Tokyo, 日本)下进行观察拍照.

(V) 氧化应激. 每个紫贻贝消化腺样品称取100~120 mg进行抗氧化状态的测定. 通过商业酶试剂盒(南京建成生物工程研究所)检测GPx、GST、SOD、CAT的活性和GSH的含量. 采用碧云天BCA试剂盒测定蛋白浓度. 酶活性和GSH含量分别表示为U/mg蛋白和mg/g蛋白.

(VI) 综合生物标志物响应(IBR). IBR的计算方法参考Beliaeff和Burgeot^[33], 并稍做改进, 具体计算过程简述如下. 将各处理组个体的生物标志物数据(A_1)与对照组数据(A_0)进行比较, 取对数以减少方差 $Y = \log A_1/A_0$. 计算各处理组中 Y 的平均值(m)和标准差(s), 将 Y 标准化为 $Z_1 = (Y - m)/s$. 计算生物标志物偏差指数 B : $B = Z_1 - Z_0$ (Z_1 为处理组, Z_0 为对照组). 对每种生物标志物的 B 值进行绝对值求和, 计算出IBR值: $\text{IBR} = \sum |B|$. 星形图中表述生物标志物偏差指数(B), 其中 A 高于0的区域表明生物标志物被诱导, 低于0的区域表明生物标志物被抑制.

2 结果与讨论

2.1 石墨烯纳米材料表征

本研究中所使用的石墨烯通过SEM和AFM进行表征(图1), 表征之前通过超声波处理15 min获得石墨烯均匀分散体. SEM图像表明石墨烯具有锋利边缘(图1(a)). AFM对石墨烯纳米材料的形貌研究表明, 所采用的石

图1 (网络版彩色)扫描电子显微镜(SEM)和原子力显微镜(AFM)表征石墨烯. (a) SEM扫描图片; (b) AFM扫描图片以及通过AFM数据绘制的石墨烯厚度和尺寸图

Figure 1 (Color online) Characterization of graphene samples. (a) SEM images of graphene; (b) AFM images of graphene with height and lateral size

石墨烯具有典型的纳米片形貌。如图1(b)所示,石墨烯厚度在1.0 nm左右,横向尺寸在0.1~0.5 μm 之间,与文献[1]报道单层石墨烯厚度一致。石墨烯类碳纳米材料的尺寸会影响其毒性,GO是石墨烯的氧化物形式[5]。文献[34]报道,尺寸较大的GO(750~1300 nm)吸附于细胞质膜上,减少其吞噬作用;而尺寸较小的GO(5~350 nm)则更易被细胞吸收。

2.2 氧化应激

研究表明,氧化损伤是石墨烯产生生物毒性的主要机制之一[35]。生物体组织细胞内各种抗氧化酶活性和抗氧化物含量,可以反映生物体的抗氧化状态和体内ROS水平,通过对它们的活性和含量进行检测,可以评估生物体受到氧化胁迫的程度[36]。如图2所示,石墨烯暴露可显著增加消化腺组织中GPx、GST、SOD和CAT活性以及GSH含量($P<0.05$)。与石墨烯暴露相似,TPP暴露可显著增加GSH含量和GST、SOD以及CAT活性($P<0.05$)。据文献[37]报道,GO(>100 mg/L)暴露后,卤虫(*Artemia salina*)体内的丙二醛(MDA)含量、总抗氧化能力、活性氧种类和SOD活性均呈剂量依赖性显著增加,说明GO的毒性作用是氧化损伤所致。有研究发现,TPP(>100 mg/L)可显著升高小鼠肝脏组织内MDA含量,降低谷胱甘肽(GSH)含量,显著影响GPx、CAT和GST等抗氧化酶活性[38]。本研究发现石墨烯和TPP在较低浓度下(0.5 mg/L)分别单独暴露均可增加紫贻贝体内GSH含量和抗氧化物活性(GST、SOD和CAT),表明石墨烯与TPP均可产生氧化胁迫。

石墨烯+TPP暴露后,紫贻贝肝胰腺内GPx、GST、SOD、CAT活性和GSH含量显著低于石墨烯单独暴露组($P<0.05$),表明联合暴露组的氧化应激水平低于石墨烯单独暴露组。研究表明,石墨烯可以通过 π - π 键和氢键强烈吸附具有相似六碳环的其他有机物,例如多环芳烃(PAH)和多溴二苯醚(PBDEs)[39,40]。本研究中,石墨烯和TPP均具有六元环,因此推测联合暴露组中氧化应激水平降低的原因可能是,TPP吸附到石墨烯表面后,改变了石墨烯的表面锐度和活性,从而降低了石墨烯对紫贻贝的氧化胁迫。

2.3 组织病理损伤

氧化胁迫可导致组织损伤、细胞膜损伤和其他毒性作用[10]。紫贻贝是一种滤食性双壳贝类,食物会先经过鳃的过滤再进入消化道。因此,鳃是与周围环境接触的第一个部位,其次是消化腺组织[41,42]。本研究检测了石墨烯和TPP暴露后紫贻贝鳃和肝胰腺的组织变化。海水和DMSO对照组中,紫贻贝鳃组织结构完整,鳃丝未出现肿胀和腐蚀的情况(图3)。TPP和石墨烯单一和联合暴露组中,鳃组织均出现病理现象,包括纤毛脱落、血细胞渗透和鳃丝肿胀。TPP暴露组中(图3(c)),血细胞渗透(IV)和鳃丝肿胀(VI)尤为明显。石墨烯暴露组中(图3(d)),纤毛脱落(V)现象严重。联合暴露组出现了鳃丝肿胀(VI)和纤毛脱落(V),但损伤程度明显低于石墨烯单独暴露(图3(e))。

石墨烯和TPP暴露后紫贻贝肝胰腺组织的病理学结果如图4所示。海水和DMSO对照组中,肝胰腺的消

图2 (网络版彩色)石墨烯和TPP暴露14 d后,紫贻贝消化腺组织中抗氧化酶活性和GSH含量的变化。(a)紫贻贝消化腺组织中GST(谷胱甘肽S-转移酶)、SOD(过氧化物歧化酶)、GPx(谷胱甘肽过氧化物酶)、CAT(过氧化氢酶);(b)紫贻贝消化腺组织中GSH(谷胱甘肽)含量。结果以平均值 $\pm$ S.D表示,*: $P<0.05$

Figure 2 (Color online) Antioxidant enzyme activities and GSH content in hepatopancreas tissues of *M. galloprovincialis* after the exposures to graphene, TPP and their combination for 14 d. (a) The antioxidant enzyme activities of GPx, GST, SOD and CAT in hepatopancreas tissues of *M. galloprovincialis*. (b) The GSH content in hepatopancreas tissues of *M. galloprovincialis*. The data are presented as the mean $\pm$ S.D, *: $P<0.05$

图 3 (网络版彩色)石墨烯和TPP暴露后紫贻贝鳃组织的病理图片(200 ×). (a) 海水对照; (b) DMSO对照; (c), (d) TPP; (e), (f) 石墨烯; (g), (h) 石墨烯 + TPP. I: 鳃上皮; II: 鳃丝; III: 纤毛; IV: 血细胞渗透; V: 纤毛脱落; VI: 鳃丝肿胀. 标尺50 μm

Figure 3 (Color online) The histopathology images of the gill after exposure to graphene, TPP and their combination (× 200). (a) Seawater control; (b) DMSO control; (c), (d) TPP; (e), (f) graphene; (g), (h) graphene + TPP. I, Gill epithelium; II, gill filament; III, cilia; IV, hemocyte infiltration; V, cilia loss; VI, gill filaments swollen. Scale bars are 50 μm

化管轮廓清晰完整, 周围结缔组织结构正常, 内壁间质组织呈“星形”结构, 没有明显的组织坏死及其他组织损伤. TPP(图4(c))和石墨烯(图4(d))单一暴露组中, 肝胰腺组织脱落(IV)、血细胞渗透(III)和上皮细胞坏死

(V)现象严重. 联合暴露组织损伤现象相对于石墨烯单独暴露组有所减轻, 出现上皮细胞坏死现象(V), 但无明显组织脱落和血细胞渗透(图4(e)).

石墨烯片层横向尺寸较大且边缘锋利(图1), 会在

图4 (网络版彩色)石墨烯和TPP暴露后紫贻贝肝胰腺组织的病理图片(200×). (a) 海水对照; (b) DMSO对照; (c), (d) TPP; (e), (f) 石墨烯; (g), (h) 石墨烯 + TPP. I: 消化管; II: 内腔; III: 血细胞渗透; IV: 组织脱落; V: 上皮细胞萎缩坏死. 标尺50 μm

Figure 4 (Color online) The histopathology images of the hepatopancreas after exposure to graphene, TPP and their combination (× 200). (a) Seawater control; (b) DMSO control; (c), (d) TPP; (e), (f) graphene; (g), (h) graphene + TPP. I, Digestive tube; II, lumen; III, hemocyte infiltration; IV, tissue loss; V, atrophy and necrosis of epithelial cells. Scale bars are 50 μm

紫贻贝呼吸和滤食过程中对鳃组织造成物理损伤, 进入消化道后可对消化道组织造成损伤^[43]. Ma等人^[34]研究发现, 以J774.A1巨噬细胞为模型的体外研究中, GO可以诱导细胞的炎症反应, 并可导致细胞死亡. 以小鼠

为研究对象, 经腹腔注射进行体内暴露后, GO可诱导腹腔局部和血液系统产生炎症反应. 我们前期的研究表明, TPP暴露7 d后, 紫贻贝鳃丝和纤毛清晰可见, 组织形态无明显变化^[43]. 本研究中TPP的暴露时间为14 d,

紫贻贝鳃和消化腺组织病理切片出现明显的损伤,说明随暴露时间的延长,TPP造成的组织损伤加重.与石墨烯单独暴露相比,联合暴露后紫贻贝组织损伤减轻,说明石墨烯吸附TPP后,改变了石墨烯的表面锐度和活性,从而减弱石墨烯对紫贻贝的组织损伤^[39].

2.4 生理指标

呼吸和排泄是贝类新陈代谢的基本生理活动,它既反映了贝类自身的生理状态,也反映了不同环境要素对贝类基础生理活动的影响^[44].CR和IR具有相同的表达趋势,图5中只对CR进行了分析.与对照组相比,石墨烯和TPP单一及联合暴露组滤水率均有下降,但无显著性差异(图5(a)).石墨烯和TPP能够抑制紫贻贝的滤水率,意味着紫贻贝的能量摄入过程受到限制.紫贻贝通过鳃进行滤食,因此鳃组织易受到海水中污染物的影响.NPs吸附在鳃表面会产生多种不利影响,如鳃病理(如增生和水肿)、呼吸毒性、氧化应激和饮食压力,这些现象都会影响贻贝的过滤和摄食^[45].在本研究中,石墨烯和TPP暴露引起紫贻贝鳃组织的病理损伤

(图3),这可能是导致紫贻贝滤水率降低的主要原因.

石墨烯和TPP单独暴露均可增加紫贻贝的排氨率,但是联合暴露后紫贻贝的排氨率低于石墨烯单独暴露组(图5(b)).石墨烯显著抑制紫贻贝呼吸,联合暴露后可降低由石墨烯引起的呼吸抑制(图5(c)).研究发现,当生物体受到环境胁迫时,生物体通常采用降低机体代谢来减少能量消耗^[46].例如,海水酸化和热胁迫暴露能够抑制厚壳贻贝硬壳蛤(*Mytilus coruscus*)的能量代谢^[21].本研究结果表明,紫贻贝为了应对石墨烯和TPP的胁迫压力,降低了体内的能量消耗,表明紫贻贝处于亚健康状态,长期持续暴露可能会对其种群维持构成潜在威胁.

生物体对环境污染的总体反应是,利用体内贮存的营养物质增加代谢需求.生物体在代谢过程中消耗的氧与排泄的氨比值(O:N),代表提供给生物体能量的蛋白质与脂肪和碳水化合物的比率^[20].O:N是表示生物利用代谢底物的一个重要指标,反映生物体在特定的环境状态下体内脂肪、糖类和蛋白质代谢的比例关系^[47].另外,O:N值的高低可以作为生物体适应环境压

图5 (网络版彩色)紫贻贝在石墨烯和TPP暴露下生理指标的响应. (a) 摄食率; (b) 排氨率; (c) 耗氧率; (d) 氧氮比率. 结果以平均值±S.D.表示, *: $P < 0.05$

Figure 5 (Color online) Response of physiological parameters of *M. galloprovincialis* after the exposures to graphene, TPP and their combination for 14 d. (a) Clearance rate; (b) ammonia excretion rate; (c) oxygen consumption rate; (d) oxygen-nitrogen ratio (O:N). The data are presented as the mean ± S.D., *: $P < 0.05$

图6 (网络版彩色)石墨烯和TPP单一及联合暴露后紫贻贝生物标志物星状图和综合生物标志物响应(IBR)。 (a) 生物标志物星状图; (b) 综合生物标志物响应(IBR)

Figure 6 (Color online) Biomarker star plots and integrated biomarker response (IBR) of *M. galloprovincialis* after the exposures to graphene, TPP and their combination. (a) Biomarker star plots; (b) integrated biomarker response (IBR)

力的指标之一^[48]。当生物体以蛋白质代谢为主时, O:N 值约为7.0~9.3; 当生物以脂肪和碳水化合物代谢为主时, O:N 值通常高于24^[49]。文献^[21]报道, 海水温度升高和海洋酸化会导致厚壳贻贝(*Mytilus coruscus*)的O:N 值降低, 表明贻贝体内蛋白质被降解。如图5(d)所示, 石墨烯暴露后, O:N 显著降低, 但与石墨烯暴露相比, 联合暴露后O:N 显著升高($P < 0.05$)。O:N 值低说明蛋白质分解代谢率要相对高于碳水化合物和脂肪的分解代谢率, 表明石墨烯暴露后对机体造成较大环境压力, 导致紫贻贝的主要供能物质转变为蛋白质, 但是石墨烯和TPP联合暴露对紫贻贝造成的环境压力低于石墨烯单独暴露。

2.5 综合生物标志物响应(IBR)

IBR指数通常用于衡量生态系统的健康状况, 并评估环境压力对生物体的影响^[50]。IBR的星图显示了紫贻贝9种生物标志物(NR、OR、IR、CR、GST、SOD、GPx、CAT和GSH)的响应(图6(a))。高水平的GSH、GPx、CAT、SOD和NR与石墨烯暴露呈正相关。表明石墨烯暴露后对紫贻贝产生显著的氧化胁迫, 增加NR。TPP暴露后产生的效应与石墨烯暴露组相一致。SOD、CAT、GST和GSH水平的降低与石墨烯 + TPP暴露呈

正相关, 石墨烯 + TPP暴露尤其显著减弱了石墨烯对NR、IR和CR的不利影响, 上述指标的变化与石墨烯 + TPP产生较低的氧化胁迫、组织损伤和生理反应有关。

石墨烯、TPP和石墨烯 + TPP处理的紫贻贝中的IBR值分别为36、26和23(图6(b))。IBR值高说明生物体遭受到的胁迫压力较大^[50]。石墨烯 + TPP暴露后IBR值低于石墨烯, 表明石墨烯 + TPP对紫贻贝的毒性小于石墨烯单独暴露, 这与抗氧化状态、生理反应和组织损伤的发现一致。

3 结论

本研究表明石墨烯和TPP诱导紫贻贝体内氧化应激, 损伤鳃和肝胰腺组织。石墨烯与TPP联合暴露可降低由石墨烯单独暴露引起的氧化胁迫和组织损伤。紫贻贝的鳃组织损伤是导致滤水率降低的主要原因。石墨烯显著抑制紫贻贝的呼吸、增加其排氨率, 但联合暴露后可降低由石墨烯诱导的这两种不利反应。联合暴露组中O:N 比显著高于石墨烯单独暴露组, 表明联合暴露对机体产生的胁迫压力较小。IBR值也表明紫贻贝对石墨烯和TPP联合暴露的生理响应低于石墨烯单独暴露。综上所述, 石墨烯和TPP联合暴露可以降低由石墨烯单独暴露对紫贻贝产生的不利影响。

参考文献

- 1 Zhang X, Zhou Q, Zou W, et al. Molecular mechanisms of developmental toxicity induced by graphene oxide at predicted environmental concentrations. *Environ Sci Technol*, 2017, 51: 7861–7871

- 2 Xie X, Kretschmer K, Wang G. Advances in graphene-based semiconductor photocatalysts for solar energy conversion: Fundamentals and materials engineering. *Nanoscale*, 2015, 7: 13278–13292
- 3 Peng Y J, Wu T H, Hsu C T, et al. Electrochemical characteristics of the reduced graphene oxide/carbon nanotube/polypyrrole composites for aqueous asymmetric supercapacitors. *J Power Sources*, 2014, 272: 970–978
- 4 Song J, Lau P S, Liu M, et al. A general strategy to create RNA aptamer sensors using “regulated” graphene oxide adsorption. *ACS Appl Mater Interfaces*, 2014, 6: 21806–21812
- 5 Shen H, Zhang L, Liu M, et al. Biomedical applications of graphene. *Theranostics*, 2012, 2: 283–294
- 6 Hu C, Hu N, Li X, et al. Graphene oxide alleviates the ecotoxicity of copper on the freshwater microalga *Scenedesmus obliquus*. *Ecotoxicol Environ Saf*, 2016, 132: 360–365
- 7 Nouara A, Wu Q, Li Y, et al. Carboxylic acid functionalization prevents the translocation of multi-walled carbon nanotubes at predicted environmentally relevant concentrations into targeted organs of nematode *Caenorhabditis elegans*. *Nanoscale*, 2013, 5: 6088–6096
- 8 Pakarinen K, Petersen E J, Alvilä L, et al. A screening study on the fate of fullerenes (C_{60}) and their toxic implications in natural freshwaters. *Environ Toxicol Chem*, 2013, 32: 1224–1232
- 9 Petersen E J, Henry T B. Methodological considerations for testing the ecotoxicity of carbon nanotubes and fullerenes: Review. *Environ Toxicol Chem*, 2012, 31: 60–72
- 10 Ou L, Song B, Liang H, et al. Toxicity of graphene-family nanoparticles: A general review of the origins and mechanisms. *Part Fibre Toxicol*, 2016, 13: 57–81
- 11 Pretti C, Oliva M, Pietro R D, et al. Ecotoxicity of pristine graphene to marine organisms. *Ecotoxicol Environ Saf*, 2014, 101: 138–145
- 12 Katsumiti A, Tomovska R, Cajaraville M P. Intracellular localization and toxicity of graphene oxide and reduced graphene oxide nanoplatelets to mussel hemocytes *in vitro*. *Aquat Toxicol*, 2017, 188: 138–147
- 13 Sanchis J, Olmos M, Vincent P, et al. New insights on the influence of organic co-contaminants on the aquatic toxicology of carbon nanomaterials. *Environ Sci Technol*, 2016, 50: 961–969
- 14 Tan L Y, Huang B, Xu S, et al. Aggregation reverses the carrier effects of TiO_2 nanoparticles on cadmium accumulation in the waterflea *Daphnia magna*. *Environ Sci Technol*, 2017, 51: 932–939
- 15 Yu Z G, Wang W X. Interaction of functionalized fullerenes and metal accumulation in *Daphnia magna*. *Environ Toxicol Chem*, 2014, 33: 1122–1128
- 16 Miao W, Zhu B, Xiao X, et al. Effects of titanium dioxide nanoparticles on lead bioconcentration and toxicity on thyroid endocrine system and neuronal development in zebrafish larvae. *Aquat Toxicol*, 2015, 161: 117–126
- 17 Yang W W, Wang Y, Huang B, et al. TiO_2 Nanoparticles act as a carrier of Cd bioaccumulation in the ciliate *Tetrahymena thermophila*. *Environ Sci Technol*, 2014, 48: 7568–7575
- 18 Fernández-Reiriz M J, Range P, Álvarez-Salgado X A, et al. Tolerance of juvenile *Mytilus galloprovincialis* to experimental seawater acidification. *Mar Ecol-Prog Ser*, 2012, 454: 65–74
- 19 Duarte C, Navarro J M, Acuña K, et al. Combined effects of temperature and ocean acidification on the juvenile individuals of the mussel *Mytilus chilensis*. *J Sea Res*, 2014, 85: 308–314
- 20 Nie H T, Huo Z M, Hou X L, et al. Comparison study on the effect of temperature and salinity on oxygen consumption and ammonia excretion in Zebra strain and wild *Ruditapes philippinarum* (in Chinese). *Acta Hydrobiol Sin*, 2017, 41: 121–126 [聂鸿涛, 霍忠明, 侯晓琳, 等. 温度和盐度突变对菲律宾蛤仔斑马蛤耗氧率和排氨率的影响. 水生生物学报, 2017, 41: 121–126]
- 21 Wang Y, Li L, Hu M, et al. Physiological energetics of the thick shell mussel *Mytilus coruscus* exposed to seawater acidification and thermal stress. *Sci Total Environ*, 2015, 514: 261–272
- 22 Fan W H, Liu T, Shi Z W, et al. Oxidative stress and physiological damage of cubic and tetrahedral Cu_2O micro/nanocrystals to *Daphnia magna* (in Chinese). *Asian J Ecotoxicol*, 2016, 11: 40–48 [范文宏, 刘通, 史志伟, 等. 立方体和八面体微/纳米氧化亚铜对大型水蚤(*Daphnia magna*)的氧化胁迫和生理损伤. 生态毒理学报, 2016, 11: 40–48]
- 23 Wang Y J, Li L S, Li Q Z, et al. Research progress on eco-physiological responses of shellfish under ocean acidification and global warming (in Chinese). *Acta Ecol Sin*, 2014, 34: 3499–3508 [王有基, 李丽莎, 李琼珍, 等. 海洋酸化和全球变暖对贝类生理生态的影响研究进展. 生态学报, 2014, 34: 3499–3508]
- 24 Wang Y J, Li N, Luo X Y, et al. Effects of multi-walled carbon nanotubes on the growth and physiology of *Microcystis aeruginosa* (in Chinese). *Asian J Ecotoxicol*, 2018, 13: 316–325 [王应军, 李娜, 罗潇宇, 等. 多壁碳纳米管对铜绿微囊藻生长及生理特征的影响. 生态毒理学报, 2018, 13: 316–325]
- 25 Marklund A, Andersson B, Haglund P. Organophosphorus flame retardants and plasticizers in Swedish sewage treatment plants. *Environ Sci Technol*, 2005, 39: 7423–7429
- 26 van der Veen I, de Boer J. Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis. *Chemosphere*, 2012, 88: 1119–1153

- 27 Shi Q, Tsui M M P, Hu C, et al. Acute exposure to triphenyl phosphate (TPhP) disturbs ocular development and muscular organization in zebrafish larvae. *Ecotoxicol Environ Saf*, 2019, 179: 119–126
- 28 Montes M O, Hanna S K, Lenihan H S, et al. Uptake, accumulation, and biotransformation of metal oxide nanoparticles by a marine suspension-feeder. *J Hazard Mater*, 2012, 225: 139–145
- 29 Hull M S, Vikesland P J, Schultz I R. Uptake and retention of metallic nanoparticles in the mediterranean mussel (*Mytilus galloprovincialis*). *Aquat Toxicol*, 2013, 140: 89–97
- 30 Della Torre C, Balbi T, Grassi G, et al. Titanium dioxide nanoparticles modulate the toxicological response to cadmium in the gills of *Mytilus galloprovincialis*. *J Hazard Mater*, 2015, 297: 92–100
- 31 Su G, Crump D, Letcher R J, et al. Rapid *in vitro* metabolism of the flame retardant triphenyl phosphate and effects on cytotoxicity and mRNA expression in chicken embryonic hepatocytes. *Environ Sci Technol*, 2014, 48: 13511–13519
- 32 Li F, Ding J W, Wu H F, et al. Interaction between graphene joint triphenyl phosphate (TPP) and the simulated phospholipid bilayer of electrochemical research (in Chinese). *Chin Sci Bull*, 2017, 62: 4285–4293 [李斐, 丁家旺, 吴惠丰, 等. 电化学方法研究石墨烯和磷酸三苯酯 (TPP)与模拟生物膜间的相互作用. 科学通报, 2017, 62: 4285–4293]
- 33 Beliaeff B, Burgeot T. Integrated biomarker response: A useful tool for ecological risk assessment. *Environ Toxicol Chem*, 2002, 21: 1316–1322
- 34 Ma J, Liu R, Wang X, et al. Crucial role of lateral size for graphene oxide in activating macrophages and stimulating pro-inflammatory responses in cells and animals. *ACS Nano*, 2015, 9: 10498–10515
- 35 Singh S K, Singh M K, Nayak M K, et al. Thrombus inducing property of atomically thin graphene oxide sheets. *ACS Nano*, 2011, 5: 4987–4996
- 36 Zhang J, Yuan L, Liu W, et al. Effects of UV-C on antioxidant capacity, antioxidant enzyme activity and colour of fresh-cut red cabbage during storage. *Int J Food Sci Technol*, 2017, 52: 626–634
- 37 Zhu S, Luo F, Chen W, et al. Toxicity evaluation of graphene oxide on cysts and three larval stages of *Artemia salina*. *Sci Total Environ*, 2017, 595: 101–109
- 38 Chen G, Jin Y, Wu Y, et al. Exposure of male mice to two kinds of organophosphate flame retardants (OPFRS) induced oxidative stress and endocrine disruption. *Environ Toxicol Pharmacol*, 2015, 40: 310–318
- 39 Wu X, Wang W, Zhu L. Enhanced organic contaminants accumulation in crops: Mechanisms, interactions with engineered nanomaterials in soil. *Environ Pollution*, 2018, 240: 51–59
- 40 Sun H, Feng R, Nan Y, et al. *In-situ* examination of graphene and graphene oxide impact on the depuration of phenanthrene and fluoranthene adsorbed onto spinach (*Spinacia oleracea* L.) leaf surfaces. *Environ Pollution*, 2018, 237: 968–976
- 41 Chandurvelan R, Marsden I D, Gaw S, et al. Impairment of green-lipped mussel (*Perna canaliculus*) physiology by waterborne cadmium: Relationship to tissue bioaccumulation and effect of exposure duration. *Aquat Toxicol*, 2012, 124: 114–124
- 42 Paul-Pont I, Lacroix C, González Fernández C, et al. Exposure of marine mussels *Mytilus* spp. To polystyrene microplastics: Toxicity and influence on fluoranthene bioaccumulation. *Environ Pollution*, 2016, 216: 724–737
- 43 Meng X, Li F, Wang X, et al. Combinatorial immune and stress response, cytoskeleton and signal transduction effects of graphene and triphenyl phosphate (TPP) in mussel *Mytilus galloprovincialis*. *J Hazard Mater*, 2019, 378: 120778
- 44 Jiao H F, Yan Q N, Zheng D, et al. Effects of temperature and salinity on oxygen consumption and ammonia excretion of muddy shellfish *Tegillarca granosa* (in Chinese). *Oceanol Limnol Sin*, 2015, 46: 1333–1338 [焦海峰, 严巧娜, 郑丹, 等. 温度和盐度对埋栖性双壳类泥蚶 (*Tegillarca granosa*)呼吸、排泄的影响. 海洋与湖沼, 2015, 46: 1333–1338]
- 45 Hu M, Lin D, Shang Y, et al. CO₂-induced PH reduction increases physiological toxicity of nano-TiO₂ in the mussel *Mytilus coruscus*. *Sci Rep*, 2017, 7: 40015–40026
- 46 Portner H O. Physiological basis of temperature-dependent biogeography: Trade-offs in muscle design and performance in polar ectotherms. *J Exp Biol*, 2002, 205: 2217–2230
- 47 Person-Le Ruyet J, Mahé K, Le Bayon N, et al. Effects of temperature on growth and metabolism in a mediterranean population of european sea bass, *Dicentrarchus labrax*. *Aquaculture*, 2004, 237: 269–280
- 48 Widdows J, Bayne B L. Temperature acclimation of *Mytilus Edulis* with reference to its energy budget. *J Mar Biol Ass*, 1971, 51: 827–843
- 49 Mayzaud P. Respiration and nitrogen excretion of zooplankton. II. Studies of the metabolic characteristics of starved animals. *Mar Biol*, 1973, 21: 19–28
- 50 Turja R, Höher N, Snoeijis P, et al. A multibiomarker approach to the assessment of pollution impacts in two Baltic Sea coastal areas in Sweden using caged mussels (*Mytilus trossulus*). *Sci Total Environ*, 2014, 473: 398–409

Summary for “石墨烯联合磷酸三苯酯(TPP)胁迫紫贻贝(*Mytilus galloprovincialis*)的生理生化响应”

Physiological and biochemistry responses of graphene combined with triphenyl phosphate (TPP) to *Mytilus galloprovincialis*

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After released into the environment, graphene nanomaterials can interact with other pollutants, due to their excellent adsorption properties. This brings changes to the environmental behavior and toxicological effects of graphene nanomaterials and other pollutants. Therefore, it is of great significance to further investigate the joint toxic effects of graphene and other pollutants. Triphenyl phosphate (TPP) is an organophosphate flame retardant and widely distributed in the Bohai sea. TPP can interact with graphene easily, as it contains three benzene rings in the molecule structure. Previous studies showed that graphene could increase the accumulation of TPP because of its hydrophobicity and durability. Due to the increasing applications of graphene and TPP, it is of great significance to study the combined toxicological effects of graphene and TPP.

Marine bivalves are important model organisms to assess the effects of nanoparticles on aquatic biota, and may play a significant role in nanoparticles uptake, biotransformation and trophic transfer through food chains. The physiological response index can be used to evaluate the effects of environmental stressors on marine bivalves. These physiological parameters not only present the key processes related to energy budget, but also reflect the physiological plasticity of animals to stress environments. As a species with high tolerance and efficient accumulation of pollutants, mussel *Mytilus galloprovincialis* has been widely used as the bioindicator for environmental monitoring and ecotoxicological research. In this study, the physiological responses of graphene and TPP on *M. galloprovincialis* were explored based on the analyses of five groups, including seawater control, DMSO control, graphene (0.5 mg/L), TPP (0.5 mg/L) and graphene (0.5 mg/L) + TPP (0.5 mg/L). The antioxidant status, physiological responses and histological changes of gill and hepatopancreas tissues were measured after 14-d exposure to graphene and TPP. The oxidative stress effects of graphene and TPP on *M. galloprovincialis* were examined by obtaining a series of enzyme activity indicators, such as the activities of catalase (CAT), superoxide dismutase (SOD), glutathione-s-transferase (GST), glutathione peroxidase (GPx) and the content of glutathione (GSH). The key physiological parameters that characterized changes in physiological response were measured, including clearance rate (CR), ammonia excretion rate (NR), oxygen consumption rate (OR) and oxygen to nitrogen (O:N). In addition, the integrated biological response (IBR) index was also calculated to compare the toxic effects of graphene, TPP and graphene + TPP on *M. galloprovincialis*.

The results showed that the exposure of graphene + TPP mitigated oxidative stress and tissue damage in mussels induced by graphene. Graphene had significant effects on the oxygen consumption and ammonia excretion ($P < 0.05$). Compared with the control group, graphene could significantly inhibit the OR of mussels and increased the NR. The NR of *M. galloprovincialis* was increased significantly after TPP exposure. Compared with the single graphene exposure, respiratory inhibition induced by graphene decreased, while oxygen-nitrogen ratio (O:N) increased after the combined exposure. It was evidenced that the combined exposure resulted in less environmental pressure on mussels compared with graphene exposure. The *M. galloprovincialis* was in a sub-health state after graphene and TPP exposure, and long-term continuous exposure could pose a potential threat to its population maintenance. In addition, IBR index showed that the combined exposure of graphene and TPP exhibited significantly lower stress on mussels, compared with the exposure of graphene and TPP, respectively.

mussel, graphene, triphenyl phosphate, joint effects, physiological responses

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